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Amjad M. Husaini, Muhammad Sohail

PII: S0168-1656(24)00120-2
DOI: https://doi.org/10.1016/j.jbiotec.2024.04.015
Reference: BIOTEC9371

To appear in: *Journal of Biotechnology*

Received date: 7 March 2024
Revised date: 22 April 2024
Accepted date: 23 April 2024

Please cite this article as: Amjad M. Husaini and Muhammad Sohail, Agrochemical-free genetically modified and genome-edited crops – towards a 'greener' green revolution and achieving the United Nation's Sustainable Development Goals, *Journal of Biotechnology,* (2024) doi:https://doi.org/10.1016/j.jbiotec.2024.04.015

Agrochemical-free genetically modified and genome-edited crops – towards a 'greener' green revolution and achieving the United Nation's Sustainable Development Goals

Amjad M. Husaini [1*] and Muhammad Sohail [2]

[1]Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Jammu and Kashmir, India

[2]Wolfson College, Linton Road, University of Oxford, Oxford, United Kingdom

[*]Correspondence: amjadhusaini@skuastkashmir.ac.in; orcid.org/0000-0003-1293-3118

Compliance with ethical standards

- Ethics approval and consent to participate: Not applicable
- Consent to publication: All authors have given consent to the publication
- Funding: The authors did not receive support from any organization for the submitted work
- Code availability: Not applicable
- Research involving human participants and/or animals: Not applicable
- Informed consent: Not applicable
- Conflict of interest: There are no competing interests to declare
- Author contributions: AMH and MS together conceived the idea, designed, and wrote the manuscript and finalized it together and approved it for publication
- Acknowledgement: AMH is grateful to the members of Genome Engineering & Sustainable Biotechnology Laboratory, SKUAST-K, for their support and cooperation, especially to Ms. Tanzeel Bashir for her helping with the figures.

Abstract

Sustainable farming on ever-shrinking agricultural land and declining water resources for the growing human population is one of the greatest environmental and food security challenges of the 21st century. Conventional, age-old organic farming practices alone, and foods based on costly cellular agriculture, do not have the potential to be upscaled to meet the food supply challenges for feeding large populations. Additionally, agricultural practices relying on chemical inputs have a well-documented detrimental impact on human health and the environment. As the available farming methods have reached their productivity limits, new approaches to agriculture, combining friendly, age-old farming practices with modern technologies that exclude chemical interventions, are necessary to address the food production challenges. Growing genetically modified (GM) crops without chemical inputs can allow agricultural intensification with reduced adverse health and environmental impacts. Additionally, integrating high-value pleiotropic genes in their genetic improvement coupled with the use of modern agricultural technologies, like robotics and artificial intelligence (AI), will further improve productivity. Such ‘organic-GM’ crops will offer consumers healthy, agrochemical-free GM produce. We believe these agricultural practices will lead to the beginning of a potentially new chemical-free GM agricultural revolution in the era of Agriculture 4.0 and help meet the targets of the United Nation’s Sustainable Development Goals (SDGs). Furthermore, given the advancement in the genome editing (GE) toolbox, we ought to develop a new category of ‘trait-reversible GM crops’ to avert the fears of those who believe in ecological damage by GM crops. Thus, in this article, we advocate farming with no or minimal chemical use by combining chemical-free organic farming with the existing biofortified and multiple stress tolerant GM crops, while focusing on the development of novel ‘biofertilizer-responsive GE crops’ and ‘trait-reversible GE crops’ for the future.

Keywords: Genetically modified organisms (GMOs); Genome edited crops (GE crops); organic agriculture; agrochemicals; Sustainable Development Goals (SDGs); food production; chemical-free; climate change

Abbreviations:

Bt: *Bacillus thuringiensis*

CGIAR: Consultative Group on International Agricultural Research

FAO: Food and Agricultural Organization

GM: Genetically modified

IFAD: International Fund for Agricultural Development

SDGs: Sustainable Development Goals

WFP: World Food Program

ZFN: Zinc-finger nuclease

TALEN: Transcription activator-like effector nucleases

CRISPR: Clustered regulatory interspaced short palindromic repeats

GE: Genome edited

Introduction

Agriculture has developed in the past three centuries through the following main phases: a) the 19th century organic or integrated farming phase; b) the 20th century mechanized, and chemical inputs-driven green revolution phase, and c) the modern chemical-driven genetically modified crop-based phase.

Historically, there were limited landraces or heirloom varieties that farmers cultivated, and later ‘artificial’ crossing was done by plant breeders to create thousands of plant varieties suited for high-input agriculture during and after the green revolution. However, with the advent of newer present-day challenges of growing human population, declining agricultural land and water resources, and climate change, these varieties are unlikely to be sustainable in the long run (for example, see (Firbank et al., 2018).

Currently, the key sources of food are plant- and animal-based – both being the constituent components of a fragile food production and supply system. Several alternative food sources have been explored (Figure 1). For example, towards the middle of the 20th century, the food industry started developing biotechnological techniques to produce varied food items under controlled conditions **(Table 1)** (Mojsov, 2016). Many such products are based on algal extracts like *Chlorella* health drinks (*Chlorella* growth factor), *Dunaliella* carotenoid-enriched oily extracts (capsules), and *Spirulina* liquid CO_2 extracts (antioxidant capsules) (Pulz and Gross, 2004). Carotenoids from algae gained commercial importance for food applications globally, even though their production is not yet cost-effective compared to synthetic ones (Ambati et al., 2019). As extract or processed in food products, *Spirulina* supports the digestive tract function as it helps maintain healthy intestinal bacteria (Pulz and Gross, 2004).

Microorganisms are considered candidates for food and feed due to their ability to use inexpensive feedstocks as carbon and energy sources for protein production and increase biomass rapidly (Tusé and Miller, 1984). Microalgae (e.g. chlorella), cyanobacteria (e.g. spirulina) and microfungus (e.g. *Fusarium venenatum*) have been studied extensively for microbial food production, while microbial cultures are used for enriching food items (e.g. *Rhizopus oligosporus* is used to ferment soya bean ‘Temph’, *Aspergillus oryzae* to saccharify rice for making alcoholic beverage ‘Sake’, and *Saccharomyces cerevisiae* is vital for winemaking, baking, and brewing) (Ritala et al., 2017). However, microorganisms have not developed into a significant food alternative due to their high nucleic acid content, slower digestibility, tedious purification processes to eliminate undesirable odours, and compounds having allergic reactions. Securing approval from agencies like the U.S. Food & Drug Administration (FDA) and European Food Safety Authority (EFSA) for food-grade microorganisms certified for safe human consumption is another major challenge for acceptance of microbial foods (Choi et al., 2022).

Manufacturing food biotechnologically (such as animal proteins and plant cells) has also been piloted successfully, but the progress in this ‘cellular agriculture’ is slow. Other alternative foods, such as genetically enhanced algae (Pulz and Gross, 2004) have not shown promise either. Additionally, because product-specific attitude, sensory appeal and health orientation significantly influence the consumers’ choice of food (Chekima et al., 2017), and the cost of production, such alternative foods are likely to remain niche. More importantly, none of the above food production methods has the capacity to be upscaled to the levels required to feed large populations; therefore, such products are unlikely to replace the conventional food sources for the foreseeable future.

Genetic engineering allows the introduction of desirable traits in plants; for example, improved yield, climate resilience, better nutrient use efficiency, disease resistance, enhanced nutritive and medicinal value (Abdallah et al., 2015; Husaini and Xu, 2016a, b; Husaini, 2010; Husaini and Abdin, 2008; Husaini et al., 2012; Husaini AM, 2010; Lucca et al., 2001; Wani et al., 2013; Ye et al., 2000; Zafar et al., 2020). Additionally, genetic technologies, such as CRISPR, can help improve plant metabolic profile by accumulating anti-inflammatory, antioxidant, antiviral, pharmacological, anti-carcinogenic, anti-allergic, and therapeutic metabolites, bringing considerable health benefits (Jiang and Doudna, 2017). Thus, this toolbox of genetic engineering technologies has immense potential for improving global food and nutritional security in a sustainable manner (Ma et al., 2015; Mahas and Mahfouz, 2018; Pyott et al., 2016; Roca Paixao et al., 2019), contributing to the vision the United Nation’s Sustainable Development Goals (SDGs) (Table 2). Here, we suggest combining organic farming practices and GM crops (assisted with modern tools such as robotics, nanotechnology and AI) (De Clercq et al., 2018; Group, 2019; Husaini, 2021, 2022; Husaini and Khurshid, 2021; Husaini et al., 2018; Jouanjean, 2019; Rose and Chilvers, 2018; Trendov et al., 2019) to create an opportunity for harnessing the benefits of a potentially new, agrochemical-free, GM agricultural revolution in the era of Agriculture 4.0 – a ‘greener’ green agricultural revolution.

Global food security and the United Nation’s Sustainable Development Goals

At a high-level conference on World Food Security in June 2008, convened by FAO, IFAD, WFP and CGIAR, 181 countries adopted a declaration that “It is essential to address the question of how to increase the resilience of present food production systems to challenges posed by climate change” (Husaini and Tuteja 2013).

Linked to this are the UN's SDGs. The UN's 2030 Agenda for SDGs represents a common framework of international cooperation to promote sustainable development. One of these (SDG-2) is to "end hunger, achieve food security and improved nutrition, and promote sustainable agriculture". It is of central importance to the entire SDG agenda, as it aims to promote healthy and sustainable diets, ensure global food security, and is intrinsically related to the environment, economy and society (Food, 2016). FAO's report in 2020 accepts that hunger and child malnutrition is a threat, that "the world is not on track to achieve the global nutrition targets and Zero Hunger, and that the situation could deteriorate if we do not act immediately boldly" (FAO and UNICEF).

Climate change is another major challenge (SDG-13) influencing agriculture production systems worldwide, adversely affecting crop growth, yield and quality. Unfortunately, the current food consumption patterns impose additional hidden costs related to health (SDG-3) and climate change (SDG-13) (FAO and UNICEF). According to FAO, IFAD, UNICEF, WFP and WHO by 2030, health costs associated with mortality and diet-related non-communicable diseases could exceed USD 1.3 trillion per annum, while the diet-related social cost of greenhouse gas emissions shall be approximately USD 1.7 trillion per annum (FAO and UNICEF). Based on a meta-analysis 70% of the studies on the impact of climate change have shown a decline in crop yields by the 2030s, with half of them projecting a reduction between 10-50% (Challinor et al., 2014).

The Global Report on Food Crises (GRFC, 2021) shows the worsening of acute food insecurity as the number of people in crisis (IPC/CH Phase 3 or above) reached a five-year high in 2020. Weather extremes were the primary drivers of the acute food insecurity situation for almost 34 million people in 25 countries in 2019, while in 2020, economic shocks (including COVID-19) became the primary driver of the food crisis with over 40.5 million people in crisis (IPC/CH Phase 3 or above) in 17 countries (GRFC, 2021).

The significance of these declarations, reports and goals became evident during the recent COVID-19 pandemic and regional military and political conflicts, which have impacted the socio-economic fabric of almost all societies, creating immense food production and supply challenges, particularly in developing societies. A future pandemic of a similar or a higher magnitude to COVID-19, or another geo-/socio-political or environmental calamity are not improbable and can hit both food production and supply chains even harder. Furthermore, the socio-economic impacts of pandemics and environmental events can quickly undo many of the development efforts made to reach the targets set for the UN's SDGs and by other global organizations (such as (FAO and UNICEF), further underscoring the need to develop resilient and high-yielding agricultural systems, sustainable during socio/geo-political, medical, and environmental calamities.

Chem-driven versus bio-driven farming

Managing plant nutrients at optimum levels is vital for sustainable crop production. It is now well-established that chemical-intensive farming is unsustainable as it has resulted in substantial ecological imbalance, as well as harming biodiversity, insect populations and human health (Gilliom, 2007; Husaini and Tuteja, 2013; Sharma and Singhvi, 2017; Udeigwe et al., 2015; Zhang et al., 2018). Thus, enhancing crop production without environmental damage is a major challenge in the 21st century (Huws et al., 2018).

Synthetic fertilizers have been used extensively to increase crop production from arable land over the past several decades (Wang and Li, 2019). While chemical fertilizers can help increase crop production, they are expensive and also harm the environment: they deteriorate soil fertility by causing an imbalance in the soil nutrients and reducing its water-holding capacity (Nosheen et al., 2021).

Nano-nutrition is another recent development in the field of nanotechnology in which nano-sized nutrients are provided for sustainable agriculture (Husaini and Khurshid, 2021). Special nanomaterials serve as a source of macro- and micro-nutrients and act as carriers to improve crop productivity (Elemike et al., 2019). Recent progress in the successful use of nano-fertilizers is very encouraging (Ditta and Arshad, 2016). However, an overemphasis on nanotechnology in agricultural activities can cause many unintended irreversible problems like environmental and unintended health safety issues (Zulfiqar et al., 2019). Nanomaterial phytotoxicity is a cause of concern as it varies with plant species, characteristics of nanomaterials and their dosages (Ma et al., 2010). Nanomaterials are very reactive because of their minute size and enhanced surface area and are a safety concern for those working in manufacturing units and farm workers who may become exposed (Yan and Chen, 2019). Thus, despite its enormous potential, this technology does have many disadvantages.

Biofertilizers – substances containing microbial cultures to enhance the supply of nutrients to plants – are a promising alternative to hazardous chemical fertilizers. Biofertilizers are eco-friendly, non-toxic, and help maintain soil structure and biodiversity (Itelima et al., 2018). Biofertilizers constitute microbial inoculants and their organic products, which improve the growth and yield of the crops by 10-40% (Husaini and Baba, 2022). These microbial inoculants colonize the plant rhizosphere and promote its growth (Agrahari et al., 2020; Souza et al., 2015). In addition to improving soil fertility and nutrient status, these protect the plants from pests and diseases, including one of the most destructive plant pathogens, *Fusarium* sp (Matsubara and Husaini, 2016; Shabir et al., 2020). Hence, biofertilizers are a promising alternative to hazardous chemical fertilizers for sustainable agriculture and global food security (Husaini, 2021).

Knowledge about soil properties, field environment, and host specificity of strains is mandatory for successfully producing and applying biofertilizers and biopesticides. Advances in metagenomics, pan-genomics, biotechnology, microbial taxonomy, and nanotechnology have significantly helped in producing better biofertilizers and biopesticides with multiple functionalities. The increasing demand for biofertilizers and biopesticides indicates a significant shift towards an eco-friendly and sustainable agriculture system (Husaini, 2014; Husaini, 2021). However, despite having clear advantages, further research efforts are needed to: identify soil-and plant specific strains; standardize biofertilizer and biopesticide compositions which are crop and trait specific; improve the existing strains using biotechnological methods (Carbú et al., 2016; Husaini et al., 2012); and further develop physical, chemical and enzymatic indicators of soil quality and health indices, which could be used to quantify and compare the impact of agrochemical-based GM crops with organically grown GM crops. Recently, the European Union enacted new regulations which are based on the latest scientific knowledge about the ecology and biology of each microorganism, which would help ease their use in plant protection. These rules align with the European Green Deal and will contribute to the Farm to Fork strategy for sustainable food production, processing and consumption.

Cultivating artificially bred crops by organic methods - prospects and limitations

The current model of agricultural intensification based on agrochemical inputs, large monocultures, and landscape homogenisation has successfully increased yields (Tscharntke et al., 2021). However, it has caused a considerable decline in global biodiversity and is harmful to human and animal health, and the environment (Bavec and Bavec, 2015). Therefore, these ‘conventional agricultural practices’ are not sustainable in the long run, leading to a strong voice favouring organic farming.

Organic agriculture is a form of farming that uses natural resources and strategies like biofertilizers (largely relying on animal manure), biological pest control, and crop rotation (Singh, 2021). It differs from traditional agriculture by excluding the use of synthetic fertilizers, pesticides, and growth regulators in crop production (Soni et al., 2022). Nevertheless, in its current form that uses traditional, artificially bred crops, organic farming has several limitations and inadequacies and cannot feed the growing global population using the same land resources. For example, while certified organic farming is claimed as an alternative to conventional farming (Bergström and Kirchmann, 2016), it can lead to considerable yield losses, making food production inefficient on the same land space (Meemken and Qaim, 2018). Additionally, widespread adoption of organic farming would cause net increase in greenhouse gas emissions due to lower crop production per unit land, and hence the need to divert forest land to meet food demand. Also, the reliance of organic farming on animal manure is a huge constraint on its sustainability.

Organic farming uses artificially bred high-yielding varieties, most of which have already reached yield plateaus. Thus, the bigger question is whether organic farming can be viable if the whole population of 8 billion (https://www.un.org/en/desa/world-population-reach-8-billion-15-november-2022) people opt for organic food. Dr Borlaug, a Nobel Laureate (1970) and considered the father of Green Revolution, answered this question when he said, "*organic agriculture can only possibly feed four billion people, I don't see two billion* (now almost 4 billion) *willing to disappear*".

A recent report on organic agriculture clearly shows that consumption and sales of organic products are mainly limited to the wealthy and affluent markets in the US and Europe (Willer, 2021). So, it seems that organic farming is getting pushed as an enterprise by the willingness of the rich to pay more and the profit motive of the farmers. Thus, despite the hype about organic products, they have remained a niche market. Perspectives have shifted over the past two decades. The pressing food and environmental challenges offer the prospect of exploring novel ideas, which should drive policymakers to prioritize health and environment over shortsighted economic benefits.

Despite the limitations of organic farming, there is no doubt that avoiding the use of chemicals in agriculture brings health and environmental benefits, and that certified organic agriculture can stop biodiversity losses and enrich local species in comparison with conventional, agrochemical-based farming (Tuck et al., 2014). It is these beneficial characteristics of organic farming, which can be harnessed in combination with those of high-yielding GM crops to increase productivity without severely damaging the environment and human health.

Cultivating GM varieties on agrochemical inputs – pros and cons

In principle, genetic modification or engineering means changing the genetic material of an organism by artificial means (Figure 2). Humans have carried out genetic improvement using methods, such as selective and crossbreeding, for thousands of years to obtain crops with desirable traits and higher yields, *de facto* making these crops 'GM'. Moreover, nature also uses genetic modification via processes such as point mutations, transposition, and gene transfer to select plants (and other organisms) with greater fitness in an ecosystem, forming the basis of adaptation and evolution.

Modern methods of genetic modification include DNA engineering using transgenesis (inter- or intra-specific gene transfer/insertion), genome editing using, for example, CRISPR, ZNF and TALEN, and site-directed or non-specific mutagenesis (Du et al., 2016; Husaini, 2010; Husaini et al., 2011; Shi et al., 2017; Zhang et al., 2021). However, not all genetic modifications are equal when it comes to public perception and lawmaking. For example, the so-called ‘upgraded’ crops developed by BASF under the trade name ‘Clearfield’ are not considered GM, even when a phenotypically identical herbicide-tolerance trait is classified as GM (Morris, 2007a; Morris, 2007b; Weidner et al., 2022). These crops are considered safe and effective despite gene flow to wild species (de Avila et al., 2021); such a gene flow is a component of the natural evolutionary process. While traditional genetic improvement by selective and crossbreeding receives almost no resistance from the public, crops improved using modern genetic modification methods are generally disapproved, largely due to decades long unfavorable propaganda. However, for the purpose of this discussion, we will refer ‘GM crops’ only to those crops that have been defined so as per the Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (OJ L 268, 18.10.2003, p. 1–23).

Modern genetic engineering techniques allow us to insert genes (transgenes) into a recipient plant genome from related or unrelated plants, bacteria, viruses, fungi, animals, and even those that have been artificially synthesized (Husaini et al., 2010). This advancement has helped overcome sexual and interspecific incompatibility, which make plant breeding by conventional approaches improbable (Husaini et al., 2011).

Genetic engineering is the fastest-adopted crop improvement technology of modern times (ISAAA, 2019). Between 1996 and 2019, the global area of GM crops increased ~112-fold, involving nearly 17 million farmers in 29 countries. The cultivation of transgenic crops has significantly increased global agricultural productivity; for example, crop yields have increased by 22%, leading to an estimated 68% increase in profit for farmers (Klümper and Qaim, 2014). Furthermore, high-value pleiotropic genes can help immensely in developing biofortified crops with resistance against multiple stresses so that the challenges of the 21st century are adequately overcome (Husaini, 2022).

Genetic engineering has led to the production of crop varieties with significantly reduced undesirable environmental footprints (Husaini and Tuteja, 2013). For example, from 1996 to 2016, there was an estimated 92.1 and 288 million kg reduction in insecticides used in GM maize and cotton, respectively (Brookes and Barfoot, 2018); GM rice and canola require less nitrogen fertilizer as they can fix nitrogen more efficiently, reduce NO_2 emissions, which has almost 300x global warming potential than CO_2 (Stern et al., 2010).

According to recent updates (Brookes and Barfoot, 2020), the adoption of GM insect-resistant and herbicide-tolerant technology has decreased pesticide application by 775.4 million kg (8.3%), thereby reducing the Environmental Impact Quotient (EIQ) by 18.5%. This technology has helped reduce fuel consumption on farms, which corresponds to the equivalent of removing ~15.27 million cars globally. The key impact of GM herbicide-tolerant technology is an aggregate reduction in both the quantity of herbicides used and the associated field EIQ values, indicating net improvements to the environment.

While GM crops are amenable to resource conservation technologies, reducing N_2O and CO_2 emissions, they still demand the use of fertilizers and other agrochemicals. One example of the use of agrochemicals in GM crops is glyphosate, which is a broad-spectrum systemic herbicide. Glyphosate is used on glyphosate-tolerant GM crops, such as soybean. There are several concerns regarding the use of glyphosate in GM agriculture, and many countries have already imposed a ban on its commercial use (Alcántara-de la Cruz et al., 2021; Bøhn and Millstone, 2019; Kudsk and Mathiassen, 2020). However, despite the absence of GM glyphosate-resistant crops in the EU, glyphosate is widely used in the EU to eliminate both annual and perennial weeds (Duke et al., 2018). Depending upon the time of the spray, glyphosate, and its main breakdown product aminomethylphosphonic acid (AMPA), can accumulate in the plants and enter the food chain. For example, the amount of glyphosate entering the food chain can be as high as 9 g/tonne of soybean (Bøhn and Millstone 2019). Thus, while cultivating glyphosate-tolerant GM crops effectively saves labour costs, as they encourage no-tillage conservation farming, preventing soil erosion and loss of nutrients (Kudsk and Mathiassen 2019), these beneficial features are largely offset by their potential adverse effect on human health, primarily due to the use of polyethyloxylated tallow amine (POE-T) surfactants admixed with glyphosate (Kudsk and Mathiassen, 2020). Apart from unwanted harmful effects on human health, environment, microbial populations and biodiversity, and the pollution caused by agrochemical-intensive agriculture, inappropriate handling and the subsequent pesticide poisoning alone account for approximately 0.3 million deaths per annum globally (Sabarwal et al., 2018). Therefore, modernizing GM agriculture by introducing environment-friendly technologies and avoiding the use of synthetic agrochemicals appears to be the way forward in the 21st century.

Cultivating agrochemical-free GM crops – moving towards the UN's SDGs in the era of Agriculture 4.0

Technological fixes, incremental changes and transformative policies are needed to significantly change food production and to develop alternative farming approaches (Husaini and Khurshid, 2021; Husaini and Sohail, 2023). A resilient, high-value sustainable food-production model based on agrochemical-free GM crops, suitable for present and future generations, must be developed as a commercially viable substitute for agrochemical-driven agriculture (Bøhn and Millstone, 2019; Kudsk and Mathiassen, 2020).

The means through which GM Insecticide Resistant technology has influenced the environment from 1996 to 2021 is through decreased insecticide use. This is strongly evident in the case of cotton, which was traditionally subjected to intensive insecticide treatment regimens against cotton bollworms. In maize, the insecticide use savings are lesser compared to cotton. The fuel savings owing to fewer spray runs in GM Insecticide Resistant maize and cotton, and the switching from conventional tillage to reduced tillage or no-tillage farming systems enabled by GM Herbicide Tolerant crops have resulted in permanent savings in CO_2 emissions. In 2018, this reduced 2456 million kg of CO_2 emission and decreased fuel use by 920 million liters. The extensive adoption of GM Herbicide Tolerant crops in North and South Americas improved weed control and reduced the need to plough soil and seed-bed preparation for weed control. This also reduced tractor fuel consumption for tillage and reduced soil erosion cut. Additionally, more carbon remains in the soil, leading to lower GHG emissions. Owing to this, it is estimated that 5606 million kg of soil carbon was sequestered in 2018, and 20581 million kg of CO_2 was prevented from getting released into the global atmosphere. From 1996 to 2018, the reduction in fuel use is estimated as 34172 million kg of CO_2 and 12799 million litres of reduced fuel use. This is equal to taking 22.65 million cars off the road for a year (Brookes and Barfoot, 2020).

The above discussion demonstrates that both organic farming methods and GM crops have their advantages and limitations, but neither alone has the capacity to meet the future food demands without severe environmental damage. Here, we propose aggregating advantageous features of organic farming and GM crops to develop farming with no or minimal chemical use (Table 2). We also propose incorporating other modern farming technologies into ‘organic GM farming’, such as AI-assisted robotics, to enhance farming efficiency (Husaini and Sohail, 2023); for example, those developed for operations like weeding, eliminating the need for herbicide sprays. There have already been attempts to making weed control herbicide-free: Mexico, a major agricultural economy, is seeking to make weed control more mechanical and less dependent on herbicides (Alcántara-de la Cruz et al., 2021). Furthermore, as metagenomics has advanced and plant-microbe interactions have been found to be immensely useful in understanding plant health (Levy et al., 2018; Schenk et al., 2012), we also propose the development of biofertilizer-responsive GM or GE crops. We can harness microbial strains for mutualistic associations beneficial to these biofertilizer-responsive GM / GE crops, many of which would be based on host-microbe selective interactions governed by chemotaxis signaling and quorum sensing (Cregger et al., 2021; Wille et al., 2019). These GM / GE crops would be biofertilizer-responsive and suitable for chemical-free agriculture, unlike the earlier chemical fertilizer responsive varieties of green revolution. There is a considerable potential of developing such GM crop varieties by expressing genes related to secondary metabolite biosynthesis for enhanced plant defense, and also, genes that can promote beneficial microbial associations in the rhizosphere. These crop varieties would have better associations with microbes by secreting root exudates such as volatile organic compounds, organic enzymes, siderophores, small secreted proteins, and terpenes (Halverson and Stacey, 1986; Middleton et al., 2021). Such crops will require less intensive assistance than conventional organic crops, and, therefore, have better productivity with significantly reduced environmental impact. Using agrochemical-free farming practices and new-age genome-edited crops (such as via CRISPR/Cas genome editing of high-value pleiotropic genes) with improved genetic and phenotypic traits offers a sustainable way to produce food with minimal environmental cost – central to the UN's SDGs.

Recently, in India – another major agricultural economy - the Indian Ministry of Environment, Forests and Climate Change gave environmental clearance to GM mustard DMH-11, and the Indian Council for Agricultural Research declared it vital for bringing self-sufficiency in edible oil in India (icar.org.in; devdiscourse.com). In the case of Bt cotton approval in India, state and cultivator interests prevailed over the precautionary logic; however, in the case of Bt brinjal (aubergine), the politics of risk dominated the question of agro-economics. According to one opinion (Herring, 2015), this shows the inherent vulnerability of science to politics. Despite reassurances from reputed scientific bodies and Nobel Laureates, perhaps for the first time in the history of any scientific controversy, these attempts were more or less ineffective at countering the fear campaign (Husaini and Sohail, 2018). Unlike agriculture, the pharma industry faces no such problem despite using the same genetic modification techniques. Thus, technical advances alone are unlikely to tackle the food and environmental crises. Political willingness to give the food and environment emergency a priority over short-term political and economic interests is critical to futuristic farming approaches.

The consumer's right of choice – a future perspective

Non-organic food labels include foods obtained from both agrochemical-driven agriculture and genetically engineered crops. Under the current regulations, GM food can neither be labelled organic nor can the GM content be used in certified organic products. So, when it comes to buying food, consumers have two choices: buy the food produced by conventional methods (agrochemical-driven plus GM) or buy more expensive and relatively infrequently available organic food. Amidst the debate about the currently offered choice between 'organic versus conventional' and 'GM versus non-GM' crops, we propose that the consumer is given a third choice to buy GM produce that has been grown using organic methods, free from agrochemical inputs, which is currently absent (Figure 3). For this, we need to create opportunities to move towards such sustainable and futuristic farming options (Table 2), and engineer crops with improved phenotypes to substitute the use of agrochemicals.

The formulation of effective policies needs multiple iterations between theory and practice, be these related to UNs' SDG targets, or the pathway each country chooses to follow based on region-specific challenges and demands. The ruling of 25th July 2018 by the Court of Justice of the European Union on organisms developed through novel genomic techniques and the news reports of farmers in India planting Bt brinjal illegally have generated a debate. A recent article declared "that there is an urgent need for a thorough, comprehensive review of the EU regulatory frameworks for GMOs that takes into account advances in knowledge and experience as well as consistency with international law" (van der Meer et al., 2021). We need a more acceptable food label, such as 'agrochemical-free GM produce' that, besides being environment-friendly and sustainable, persuades farmers to grow GM crops without agrochemical inputs.

Furthermore, given the currently available genome modification and editing toolbox, it should now be possible to develop 'trait reversible GM crops' for food producers and consumers who fear ecological damage by GM crops. Of particular interest for this should be newly developed drug-inducible CRISPR/Cas systems like Tet-On/Off system, Cre-dependent system, chemically induced proximity system, and destabilizing domain-mediated protein degradation systems (Shaw et al., 2022; Zhang et al., 2019). This inducible system will have both the transgene/cisgene as well as an inducible genetic system to precisely inactivate or knockout the transgene/cisgene when required, simply by exposing it to the inducer molecule.

Indeed, we need to understand the public's willingness to purchase chemical-free GM produce and legislate to create new food labelling. This can be achieved by presenting concrete scientific evidence of the benefits of GM produce to the public, quashing the decades-long negative propaganda, and the lobbying of lawmakers. Only then we will be able to harness the benefits of a potentially new 'agrochemical-free GM agricultural revolution'. Undoubtedly, such farming practices will increase productivity, they will also reduce land and air pollution, providing a cleaner and healthier environment for future generations.

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Table 1: Alternative food options other than plant and animal kingdoms: the table shows pros and cons of these options for human use and consumption.

Food option	**Advantages**	**Disadvantages**	**References**
Algae	• Low nucleic acid content • Rich in carotenoids, lipids, fatty acids, proteins, carbohydrates, amino acids, hydrocarbons, etc. • Use of no-cost CO_2 and sunlight as primary substrates • Simple cultivation methods and faster growth rates	• Low digestibility as human beings cannot digest the cellulose component of the algal wall due to the absence of cellulase enzyme • Costly recovery methods for unicellular algae, and serious risk of contamination • Need to maintain warm temperatures, plenty of sunlight and CO_2	(Ambati et al., 2019); (Nasseri et al., 2011); (Ukaegbu-Obi, 2016); (Singh et al., 2019); (Nangul and Bhatia, 2021); (Mondal et al., 2012)
Bacteria	• Ability to grow on a variety of raw materials, from carbohydrates to liquid hydrocarbons • Higher protein content and favourable composition • Higher growth rate than yeast, algae and fungi • Short generation and doubling times	• Careful evaluation for endotoxin production is essential, especially in the case of gram-negative bacteria • Due to its small size and low density, harvesting from the fermented medium is difficult and costly • Limited use due to poor public acceptance of bacteria as food • High nucleic acid (especially RNA) content relative to yeast and fungi which must be reduced	(Singh et al., 2019); (Nangul and Bhatia, 2021); (Srividya et al., 2013); (Nasseri et al., 2011); (Ukaegbu-Obi, 2016); (Kuhad et al., 1997)
Yeast	• Good source of minerals and vitamins (B, E and pro-vitamin D) • Has the ability to grow at acidic pH and has a low risk of contamination • Widely accepted and used as a single cell protein • Ease of harvesting due to larger cell size and flocculation abilities • Lower nucleic acids than bacteria. • Positive health effects due to ß-glucans and α-mannans	• Methionine supplementation may be required to overcome S-containing amino acid deficiency of its proteins • Lower growth rate than bacteria • Lower protein content than bacteria	(Singh et al., 2019); (Lapeña et al., 2020); (Kuhad et al., 1997); (Nasseri et al., 2011); (Mondal et al., 2012); (Ukaegbu-Obi, 2016)
Filamentous fungi	• Mycoproteins taste like meat, and are used as an alternative to the conventional animal proteins • Ease of harvesting • Lower capital and processing costs compared to yeasts and bacteria • Produce several polysaccharide hydrolyzing enzymes that allow them to grow on lignocellulosic and other complex polymeric raw materials • Positive effect on blood cholesterol and glycemic response	• The strains need thorough evaluation for mycotoxin production • Contamination by yeast is frequent if sterility is not properly maintained • Slower growth rates than bacteria and yeast • Lower protein content than bacteria and yeasts • Cell wall digestibility is a problem for monogastric animals • Some allergic reactions have been reported	(Singh et al., 2019); (Souza Filho et al., 2019); (Nasseri et al., 2011); (Kuhad et al., 1997)

Table 2: The various options available for crop production based on seed type and farming methods

Options	**Seed Type**	**Farming Type**	**Productivity**	**Positive impact on UN's SDGs**#	**Remarks/limitations**
Organic agriculture (Option 1)	Heirloom seed	Organic farming	Very low	GOALs 3, 6, 12, 14 and 15	Compatible with 18th century needs
Conventional agriculture (Option 2)	High-yielding seed varieties (HYV)	Agrochemical-based farming	High	GOALs 2 and 8	Environmentally unsustainable
Conventional agriculture (Option 3)	GM seed[1,2]	Agrochemical-based farming	High	GOALs 2, 3, and 8	Relatively lower environmental footprint
Agrochemical-free GM agriculture (Option 4)	GM seed[1,2,3]	Agrochemical-free farming	Moderate to high	GOALs 2, 3, 8, 6, 12, 14 and 15	Environmentally friendly and sustainable

[1] Category 1: Traits for imparting stress tolerance against salinity, drought, flooding, cold, heat, insect pests, fungal pathogens, bacterial diseases, viral diseases, *etc*.
[2] Category 2 traits: Quality enhancing traits like delayed-ripening, better nutritional profile (vitamin A, iron, omega 3, phytase, mineral content), prolonged storage and shelf-life, *etc*.
[3] Category 3: Multi-trait pyramided plants with better quality traits, higher productivity, biofertilizer-responsiveness, water- and mineral-use efficiencies, *etc*.

GOAL 2: Zero Hunger*;* **GOAL 3:** Good Health and Well-being; **GOAL 8:** Decent Work and Economic Growth; **GOAL 6:** Clean Water and Sanitation; **GOAL 12:** Responsible Consumption and Production; **GOAL 14:** Life Below Water; **GOAL 15:** Life on Land

#THE 17 GOALS | Sustainable Development (un.org) (FAO and UNICEF; Food, 2016)

Figure legends:

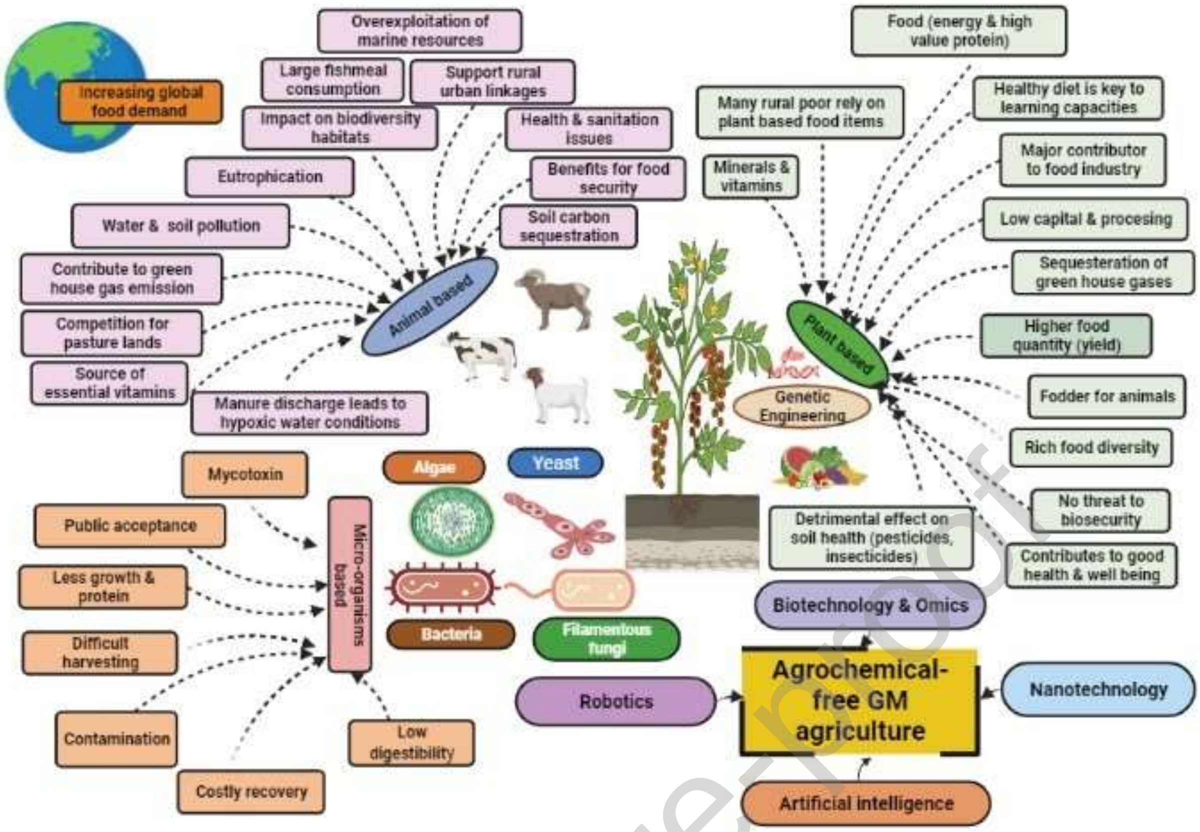

Figure 1: The available food options and their main characteristics; Animal-based nutrition is not sustainable as, among other things, it harms the environment due to air, water and soil pollution, causes competition for pastureland, and adversely affects biodiversity. Microbial-based nutrition cannot meet the growing nutritional demands owing to limited production and less public acceptance. Plant-based nutrition, while helping in the sequestration of greenhouse gases and providing fodder for animals, is a more affordable and primary source of human nutrition and a contributor to the food industry.

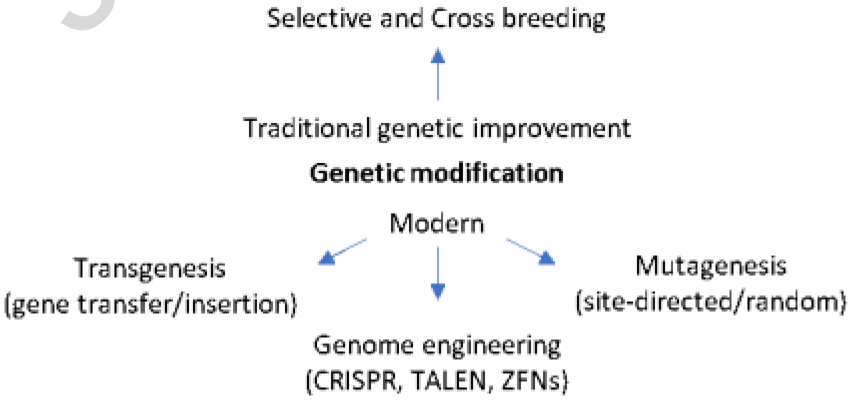

Figure 2: Key traditional and modern genetic modification/improvement methods: Genetic modification has traditionally been done by selective crossbreeding methods, while the modern

methods involve transgenesis, mutagenesis, and genome editing using Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR) with their associated protein 'Cas'.

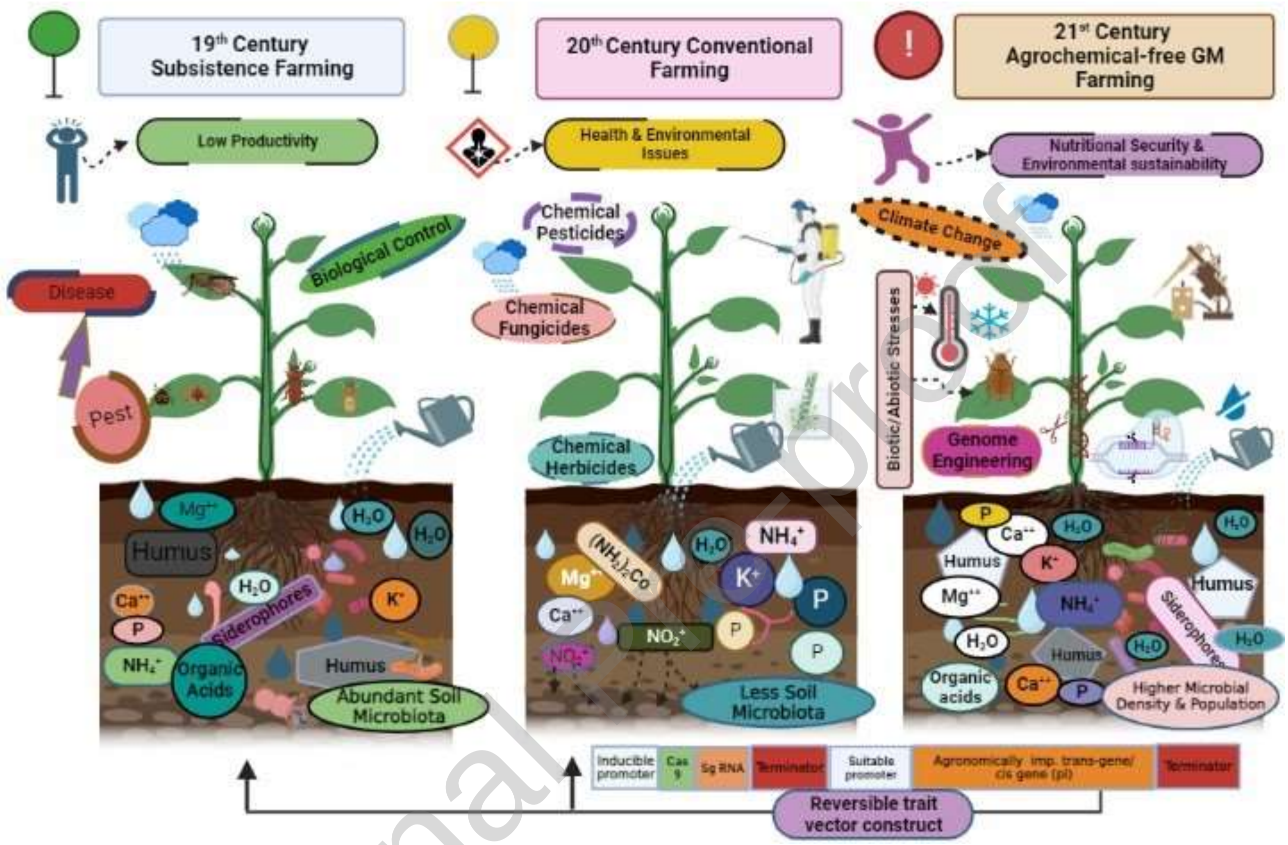

Figure 3: A schematic depiction of the major farming options under soil-plant-weather continuum from 19th to 21st century: The 19th-century subsistence farming, though environment-friendly due to organic manures and biological control, had lower productivity due to increased diseases, pests and abiotic stresses. The 20th-century conventional farming, though highly productive due to chemical-based fertilizers, pesticides, and fertilizer-responsive high-yielding crop varieties, was detrimental to the environment and human health. The 21st-century Agrochemical-free GM farming shall have to integrate organic agricultural inputs with genome-engineered crops to ensure nutritional security and environmental sustainability under the adverse climate-change imposed challenges. It may be possible to develop 'trait-reversible genome-modified crops' using genome engineering, enabling such crop varieties to be restored back to the exact same genetic state as in wild-type before the modification.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Non-organic food labels include foods from agrochemical-driven agriculture and genetically engineered crops.
- Under the current regulations, GM food can neither be labelled organic nor can the GM content be used in certified organic products.
- Not all genetic modifications are equal when it comes to public perception and lawmaking.
- Despite the hype about organic products, they have remained a niche market as organic farming is artificially sustained as an enterprise by the willingness of the rich to pay more and the profit motive of the farmers.
- Both organic farming methods and GM crops have advantages and limitations, but neither can meet future food demands in isolation.
- Modernizing GM agriculture by introducing robotics and AI-based environment-friendly technologies and avoiding synthetic agrochemicals is the way forward for a ‘greener’ future.
- Developing biofertilizer-responsive GM crops can make GM agriculture more environment-friendly, and these should be designed based on selective host-microbe interactions governed by chemotaxis signalling and quorum sensing
- Novel GM crop varieties should possess genes related to secondary metabolite biosynthesis for enhanced plant defence, and genes that promote secretion of useful root exudates (volatile organic compounds, organic enzymes, siderophores, small secreted proteins, and terpenes) for building beneficial microbial associations in the rhizosphere.
- For those who fear ecological damage by GM crops, it should now be possible to develop ‘trait reversible GM crops’, as it would help build confidence among the vacillating common people, who are overly cautious in applying the precautionary principle.

Ameliorating the effects of multiple stresses on agronomic traits in crops: modern biotechnological and omics approaches

Article *in* Molecular Biology Reports · December 2023

DOI: 10.1007/s11033-023-09042-8

4 authors:

Syed Anam Ul Haq
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Tanzeel Bashir
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Thomas H Roberts
The University of Sydney

Amjad M. Husaini
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Molecular Biology Reports (2024) 51:41
https://doi.org/10.1007/s11033-023-09042-8

REVIEW

Ameliorating the effects of multiple stresses on agronomic traits in crops: modern biotechnological and omics approaches

Syed Anam Ul Haq[1] · **Tanzeel Bashir**[1] · **Thomas H. Roberts**[2] · **Amjad M. Husaini**[1]

Received: 15 December 2022 / Accepted: 13 October 2023 / Published online: 29 December 2023

Abstract

While global climate change poses a significant environmental threat to agriculture, the increasing population is another big challenge to food security. To address this, developing crop varieties with increased productivity and tolerance to biotic and abiotic stresses is crucial. Breeders must identify traits to ensure higher and consistent yields under inconsistent environmental challenges, possess resilience against emerging biotic and abiotic stresses and satisfy customer demands for safer and more nutritious meals. With the advent of omics-based technologies, molecular tools are now integrated with breeding to understand the molecular genetics of genotype-based traits and develop better climate-smart crops. The rapid development of omics technologies offers an opportunity to generate novel datasets for crop species. Identifying genes and pathways responsible for significant agronomic traits has been made possible by integrating omics data with genetic and phenotypic information. This paper discusses the importance and use of omics-based strategies, including genomics, transcriptomics, proteomics and phenomics, for agricultural and horticultural crop improvement, which aligns with developing better adaptability in these crop species to the changing climate conditions.

Keywords Stress · Omics · Biotech crops · Phenomics · Genomics · Proteomics · Transcriptomics

Introduction

Food security is "when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food in order to meet their dietary needs and food preferences for an active and healthy life" [1]. The ever-increasing global population is exerting pressure on agricultural systems worldwide [2]. Production of almost all major cereal crops (maize, rice and wheat) has plateaued, involving many other threats to food production systems, including chaotic urbanisation (which affects agricultural acreage) and destructive weather patterns. Furthermore, crop plants are frequently subjected to many environmental stresses. Stress resistance is a complex genetic trait, and plants rarely endure only one type of stress at a time, and dealing with multiple stresses simultaneously is challenging [3]. These circumstances pose a significant threat to the nutritional and food security of the world's increasing population, which is expected to reach 9.7 billion by 2050 [4], necessitating a 70–85% increase in agricultural production.

In both tropical and temperate zones, floods, intense temperatures, persistent droughts and late-spring frosts are becoming highly prevalent [4, 5]. Although adopting an upgraded agricultural regime has helped ameliorate these concerns via the deployment of high-yield and high-resistant cultivars, future food demands, crop yields and land use require immediate consideration. Climate has always been impacted by changes in the cryosphere, biosphere, hydrosphere and various atmospheric and interacting elements. According to the United Nations estimates, increasing agricultural production by 75% by 2050 shall be required to feed the entire world's population [6]. Meeting this challenge will be more difficult if climate change melts parts of the Himalayan glaciers, jeopardising 25% of Asia's worldwide food production by affecting water availability [4, 7].

Syed Anam Ul Haq and Tanzeel Bashir have equal authorship.

✉ Amjad M. Husaini
amjadhusaini@skuastkashmir.ac.in

[1] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, SKUAST-K, Shalimar, Srinagar, Jammu and Kashmir 190025, India

[2] Plant Breeding Institute, School of Life and Environmental Sciences, Faculty of Science, Sydney Institute of Agriculture, The University of Sydney, Eveleigh, Australia

It is commonly acknowledged that human activities have a great impact on global climate change. Current patterns in human population growth and food consumption indicate that global food demand will continue to rise for at least another 40 years [8]. Breeders are confronted with new complications in these scenarios. They must identify traits that will ensure higher and consistent yields under dynamic environmental circumstances, develop lines that possess resistance (or resilience) to various emerging biotic and abiotic challenges, as well as satisfy customer demands for quality and more nutritious meals [9]. The sum total of features related to the capacity of a product to attain its necessity criteria is termed 'quality' [10]**.** In practical terms, quality represents the standards necessary to meet the wants and expectations of the consumer**.** The set of food properties that people deem acceptable make up food quality [11]. Food quality includes a lack of 'risk factors' concerning common safety requirements, along with product adherence to specifications of identity and nutrition. Since the fundamental goal of eating is to satisfy nutritional demands, nutritional requirements are of utmost importance.

Widening the pool of genetic diversity to discover novel variations or create new crop variation, along with understanding the genetics of simple and complex traits and then efficiently introgressing this variation into new elite cultivars, are all strategies to achieve these goals [12]. The focus today must not only be on expanding food availability but also on enhancing food quality. Considering the above, this paper attempts to address food production challenges under multiple stress conditions and amelioration strategies to tackle this issue successfully. We discuss multi-omics techniques and their applications to improve agronomic traits and enhance stress tolerance in important crop species. We suggest that using all these omics techniques can serve as a foundation for improving the genetic background of crops, enhancing their yields and crop tolerance to environmental stresses.

Stress-resilient agriculture can be sustained by developing 'Biotech crops'

Most crop plants thrive in suboptimal environmental circumstances that limit their genetic potential in terms of growth and reproduction [13]. Stress is typically characterised as an external force that deleteriously impacts a plant. Plants are subjected to multiple stresses on a regular basis [3, 14]. In a matter of minutes, environmental factors like air temperature, in both natural and agricultural conditions, can become stressful [15]. Factors like soil mineral deficiency can take months to become stressful, whereas soil water content can become stressful in days to weeks [16].

Biotic and abiotic stresses are the two basic types of stresses that plants endure. Drought (water scarcity), excess water (flooding), temperature extremes (freezing, chilling and heat), salt, and mineral toxicity all have negative impacts on crop yield, growth, development and seed quality. Insects, viruses, fungi, bacteria, arachnids, nematodes and weeds are among the biological organisms that make plants suffer from biotic stress. Pathogenic organisms deplete the availability of nutrients to their hosts, resulting in lower yields or even plant death. According to climate projection models, average surface temperatures are estimated to rise by 3–5 °C in the coming 50 years, wreaking havoc on global agricultural systems. Unfavourable environmental conditions in crop plants account for the enormous discrepancy between potential and actual harvest yields worldwide [17]. The environment changes constantly and plants must preserve their inherent potential to survive within their ecological niche through natural genetic variation and different adaptive mechanisms [18]. Abiotic stress factors substantially impact worldwide agricultural production and are responsible for a 50% reduction in average yields [19]. Changing climate will impact pests and pathogen habitat ranges, with rising temperatures promoting pathogen transmission and negatively influencing crop yields and nutritional quality [4, 20]. Losses due to pests range from 20 to 40% of total agricultural production globally [21].

Demand for stress-tolerant crop types has risen dramatically due to adversely dynamic climatic circumstances and growing pressure on global food production due to the population explosion [22]. The effect of these adverse challenges can be moderated to some extent through the development and cultivation of 'biotech crops' resilient to climate extremes [4]. Furthermore, accomplishing the United Nation's Sustainable Development Goals emphasises the need to explore newer approaches to agriculture, like cultivating GM crops organically, assisted by robotics, nanotechnology and artificial intelligence [23, 24]. While organic-GM agriculture will deliver agrochemical-free food, technological assistance will provide a route to efficient and precision agriculture, leading to the environmentally sustainable growth of the agriculture industry [2].

'Omics' tools are imperative for developing 'Biotech crops'

Plants are sessile; therefore, they have evolved different strategies for adapting and growing in changing environmental circumstances [25]. Biological responses of plants to stress include changes in cell membranes, cell cycle, cell division, metabolism (e.g., build-up of osmotically active substances) and cell wall architecture. At the molecular level, tactics comprise changes in gene expression, starting with transcriptional regulation up to mRNA processing, followed by translation, protein modification and protein turnover [26]. Stress-related transcriptional regulation changes the transcriptome of plants when exposed to stressful environmental circumstances [27]. These transcriptionally regulated genes play roles in different functions, viz., signalling molecules, translation, transcription, metabolism and responsiveness to stress [28]. The suffix ‘ome’ refers to the study of these processes under the domains of genome, transcriptome, proteome metabolome and phenome [29].

Over the past few decades, omics approaches have emerged as a strategy of choice for examining the genetic and molecular basis of crop biology [27, 30]. Application of omics has resulted in huge amounts of data being produced, which has improved our understanding of (a) morphological and growth patterns, (b) the genetical underpinning of various qualitative and quantitative traits, (c) expression of genes and (d) mechanisms that explain how complicated interactions between genes, proteins, and metabolites influence phenotype (Fig. 1).

High-throughput data analysis pipelines, sophisticated analytical tools and large-scale computational capabilities have been developed due to the widespread use of omics [27, 31–34]. Rapid improvements in ‘omics’ technologies have opened up the possibility of creating new datasets for crop species. Combining genomic and functional omics data with genetic and phenotypic data results in discovering genes and pathways accountable for important agronomic features [35, 36]. High-throughput genotyping approaches allow for the screening of large-scale germplasm collections to discover new alleles from several sources, resulting in a significant increase in the variation accessible for breeding [37]. In short, comprehensive multi-omics approaches provide a framework for developing novel biotech crops.

Genomics

The field of genomics focuses on the structure, function, evolution, mapping, epigenomic, metagenomic and genome-editing aspects of genes and genomes. Genomics plays a crucial role in better understanding genetic variation, increasing crop breeding effectiveness and ultimately leading to the genetic improvement of crop species. The underpinnings of modern genomics were laid by detecting DNA markers and existing polymorphisms inside a specific sequence of DNA [38]. Non-PCR-based techniques, such as restriction fragment length polymorphisms (RFLP), detect DNA polymorphism by producing a distinct DNA fragment profile when a labelled DNA probe is hybridized to a Southern blot of DNA that has been digested by restriction enzymes. PCR-based methods detect markers like single nucleotide polymorphisms (SNPs), amplified fragment length polymorphisms (AFLP) and random amplified polymorphic DNA (RAPD).

SNPs are single nucleotide variations in an individual’s genome. The SNP can be carried out by sequencing genomic PCR products obtained from various individuals [39]. Moreover, genome-wide linkage mapping and genome-wide association studies (GWAS) are used to understand and study multiple traits in crops. These methods can link complex phenotypes and genotypes, revealing variations linked to traits and revealing correlations between genetic variants/phenotypes in a population.

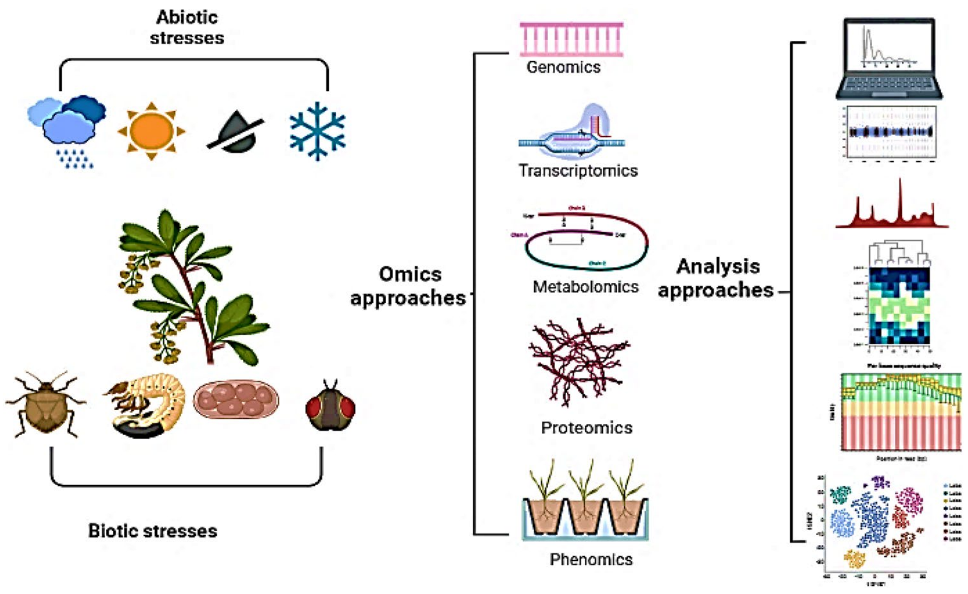

Fig. 1 Different omics approaches for multiple stress tolerance in crop plants. Multiple stresses impact the growth and development of plants negatively. The modern ‘omics’ based approaches give insight into the biology of such interactions and help better understand the ways and means to address these challenges. ABPP is an elegant technique that has taken proteomics to the next level by introducing a large and unique probe library and extensive knowledge on enzyme profiling

GWLM (genome-wide linkage mapping)

GWLM is a potent crop improvement technique that can be used to identify and characterize genetic areas linked to desired traits. It entails identifying the co-inheritance patterns of genetic markers across the entire genome and mapping them with the desired attribute. Many economically important traits are quantitative in nature, such as stress tolerance, grain yield and disease resistance [40]. When looking into the genetics of a quantitative trait, segregating populations of hundreds of individuals is required. Based on the availability of resources and objectives pertaining to the research study, various kinds of segregating populations are developed like the F2 population, advanced intercross recombinant inbred lines (AI-RIL), doubled haploid (DH) populations, near-isogenic lines (NILs), recombinant inbred lines (RILs), heterogeneous inbred family (HIF), backcross inbred lines, as well as multiparent advanced generation intercross (or MAGIC) [41].

GWLM is the most widely used methodology for determining genes that underpin important traits. In rice, GWLM has been utilized to identify genes associated with seed vigor [42]. GWLM has aided in the identification of grain quality traits in sorghum [43]. Wheat breeding programmes have used GWLM to identify loci controlling the size and shape of grain, plant height and yield [44–46]. Overall, the available literature demonstrates the efficacy of GWLM in identifying genomic regions associated with desirable traits. This knowledge can be harnessed to accelerate breeding programs, develop improved crop varieties and enhance agricultural productivity and sustainability.

GWAS (genome-wide association studies)

The genetic architecture of complex traits must be understood to grasp its biology. Most agricultural and evolutionary significant traits are complex, influenced by many genetic loci, environmental factors, and their interactions. Because of breakthroughs in sequencing technology, GWAS has become popular and a concerted effort for resequencing is undertaken after the reference genome is assembled or when a high-density genotyping array becomes available [47]. GWAS has evolved from a promising new tool to a robust and widely used technique for analysing complex traits in plants (Table 1). Natural populations overcome the constraints of the GWLM by boosting mapping precision and minimising research time [48].

A wide variety of germplasm is used in GWAS to identify marker-trait associations (MTA) and evaluate the degree of linkage disequilibrium (LD) among polymorphic markers. It is important to note the beneficial relationship between GWAS and genome editing. Better genome editing allows validating gene function across various genetic backgrounds, which can help guide GWAS research. GWAS is a vital technique for identifying the genes that underlie complex traits and provides opportunities for genome editing to create synthetic alleles. The creation of genetically modified plants with particular alleles known to affect desirable traits like drought resistance, yield and nutritional quality could also be accomplished using the biological understanding gained from combining GWAS with a wide range of genomic, phenomics, biotechnological and data analysis tools in crop species.

GWAS is crucial for improving agronomic traits and enhancing crop tolerance to environmental stresses. It identifies genomic regions involved in genotype-by-environment interactions, allowing for quantitative and qualitative trait improvement in realistic natural or agronomical conditions [66]. Genome-wide associations were used to predict SNPs in sorghum and maize, predict drought stress and drought-responsive traits using environmental variables, and identify numerous drought-responsive transcription factors [67, 68].

GWAS can be used to assess genotype performance and genomic region contribution, accelerating breeding for drought-prone environments. A study using GWAS identified 48 QTLs for maize crop yield under heat and water stress, revealing patterns and hypotheses for mechanisms and candidate genes underlying each QTL [69]. A GWAS on 121 spring barley accessions and a 9 K SNPs chip identified two drought-specific genes (HORVU2Hr1G091030 and HORVU2Hr1G091170) on chromosome 2H, affecting spikelet and final grain number under drought stress. These genes are highly expressed in spikelet, grain, spike and leaf organs, offering valuable resources for functional characterization and drought tolerance enhancement in barley cultivars [65]. A study mapped QTL for plant height (PH) and ear height (EH) in maize, revealing that multiple genes with low effect strongly control this variation. It helped in understanding the underlying mechanism of height in maize [53].

Maize oil, rich in polyunsaturated fatty acids, is valuable for human food, animal feed and bio-energy. A complementary pathways analysis focusing on cumulative SNP-trait associations was performed, revealing pathways and networks for precise and efficient genetic improvement in high-oil maize [55]. A GWAS study found potential associations between floret fertility, assimilate partitioning and spike morphology in 210 European winter wheat accessions. Shared QTL identified genes for carbohydrate metabolism, phytohormones and floral development. The study suggested a genetic network underlying floret fertility and related traits, potentially identifying determinants for improved yield performance [49]. In another study, GWAS identified QTL for sorghum protein, fat and starch. Moreover, RNAseq data identified candidate genes, with the alpha-amylase 3 gene being a strong candidate for protein and fat variation [58]. GWAS was conducted on sorghum grain size to understand

Table 1 Application of genome-wide linkage mapping and genome-wide association Studies for understanding agronomically valuable traits and stress tolerance in major crops

Crop	Mapping technique	Trait or gene examined	Remarks	Reference
Triticum aestivum	GWAS	Fertility of floret, partitioning of assimilate and morphology traits of spike	• Thirty-eight traits for assimilate partitioning and spike morphology and sixteen fertility traits were quantified in 210 European winter wheat accessions • A genetic network nominating the determinants for floret fertility traits was proposed for improved yield performance	[49]
		Spikelet number	• Genetic basis of total spikelet number, spike length and flowering time was dissected in a panel of 518 European winter wheat varieties • The study identified a 1,457-bp long gene *TaAPO-A1* as a likely candidate gene for total spikelet number in wheat	[50]
	GWLM	Size and shape of grain	• An integrated SNP and SSR high-density genetic linkage map was constructed using recombinant inbred lines and an Illumina Infinium 9 k SNP chip • QTL mapping of thousand-grain weight, grain length, grain width and grain thickness traits was done, and pleiotropic effects were detected for several QTL loci of grain shape and size	[44]
		Plant height	• The purpose was to identify markers linked to Rht24 (plant height gene) • Wheat genomic database and a 660 K SNP chip were used to show that Rht24 is an important locus for plant height and grain weight	[45]
		Height & yield	• The study attempted to identify QTLs for important genetic traits useful for wheat breeding • Ten pleiotropic QTL clusters for yield traits and eight QTLs for kernel weight, plant height and kernel number per spike were identified	[46]
Oryza sativa	GWLM and GWAS	Seed vigour	• The study aimed to identify QTLs for seed vigour in rice • Nine loci qGI-6, qGI-7, qGI-9, qGI-10, qGP-9, qVI-7, qVI-8, qVI-6–1 and qVI-6–2 responsible for seed vigour were identified using a high-density bin map	[42]
	GWAS	Stress tolerance	• OsSTL1 (Oryza sativa salt tolerance level 1) and OsSTL2 (Oryza sativa salt tolerance level 2) were identified as candidate genes for salt tolerance • Pyramiding of the superior alleles for salinity stress can help develop new varieties with improved tolerance	[51]
		Architecture of plant	• *N*-acetyl glucosamine transferase or rice *SPINDLY* (*OsSPY*) gene was identified to regulate rice architecture by suppressing gibberellin signaling in rice	[52]

Table 1 (continued)

Crop	Mapping technique	Trait or gene examined	Remarks	Reference
Zea mays	GWLM and GWAS	Ear and plant height	• QTL mapping detected twenty-one QTLs for plant height and ear height • GWAS identified forty-one SNPs • The study showed that multiple genes with low effects control maize height	[53]
		Size of male inflorescence	• Sixty-three QTLs were identified for tassel primary branch number and sixty-two for tassel length through linkage analyses in eight thousand inbreds, including both linkage and association populations of maize	[54]
	GWAS	Lipid biosynthesis	• The study aimed to elucidate the biochemical pathways, metabolic networks and associated genes contributing to oil-related traits • The acyl-lipid-associated pathway identified in the study can be useful for improving maize oil content	[55]
		Root morphology	• Genome-wide association mapping and gene expression analysis identified thirty-four QTLs associated with seedling root traits, and five DEGs as candidate genes underlying seedling root development in maize	[56]
	GWLM	Northern leaf blight resistance	• Twenty-nine QTLs were identified for resistance against northern leaf blight disease using five thousand maize inbred-line nested association mapping population • Multiple candidate genes for plant defense, including receptor-like kinase genes, were identified	[57]
Sorghum bicolor	GWAS	Composition of kernel	• The study identified promising sources of genetic material, QTLs and candidate genes for manipulating sorghum grain composition	[58]
		Plant architecture	• This study identified the association of novel genomic regions, especially gibberellin genes Ent-kaurene synthase (KS3) and Gibberellin 2-oxidase 5 (GA2ox5), with plant architecture traits like leaf angle, stem circumference, internode number, tiller number, seed number, panicle exsertion, and panicle length	[59]
		Grain size	• The genetic architecture of grain size was studied using GWAS in two large populations, and gene Grain Size 3 (SbGS3) was reported to control grain size variation in sorghum • *SbGS3* showed high expression in early inflorescence, indicating its role in grain development	[60]
	GWLM & GWAS	Grain quality traits	• Multiple sorghum mapping populations were phenotyped for grain quality traits like amylose, starch, crude protein, crude fat, and gross energy • Twenty-five grain quality QTLs were identified in different genetic backgrounds	[43]

Table 1 (continued)

Crop	Mapping technique	Trait or gene examined	Remarks	Reference
Hordeum vulgare	GWLM	Length of awn	• Multiparent mapping population derived from cultivar Morex and four genetically diverse wild barley lines were used to detect quantitative trait loci (QTLs) for awn length • Twelve QTLs were identified, and one QTL was fine-mapped to identify candidate genes in NILs by RNA-seq	[61]
		Plant height	• One-hundred eighty-two doubled haploid lines were grown in six different environments to identify QTLs for plant height and investigate QTL × environments interaction • Identified a novel QTL for plant height that expressed consistently in the six environments and can be used in breeding programs	[62]
	GWAS	Nitrogen use efficiency	• Single nucleotide polymorphism (SNP) and diversity arrays technology (DArTseq) markers were used to detect marker-trait associations with nitrogen use efficiency (NUE) related traits like plant height, root length, shoot dry weight, root dry weight, number of tillers, and number of leaves at the seedling stage • Sixty-six significant marker-trait associations (MTAs) conferring high NUE were identified • High-affinity nitrate transporter 2.7 (HvNRT2.7) gene was identified as a promising candidate for root and shoot dry weight	[63]
		Response of photoperiod	• The study aimed to detect QTL underlying natural variation of pre-anthesis stages/sub-phases based upon differences in photoperiod response in a worldwide spring barley collection • New genetic network model for each photoperiod group was proposed, which included several newly identified genes belonging to different heading time pathways in barley	[64]
		Grain yield and Spikelet number under drought stress	• The study aimed to identify the genetic basis of drought tolerance at different developmental and growth phases in one-hundred twenty-one spring barley accessions • Two candidate drought-specific genes were highly associated with spikelet and final grain number per spike under drought stress	[65]

its genetic architecture. Results showed that genes with opposite effects on grain number were less likely to overlap with the grain size QTL. It facilitated the identification of genetic regions related to the genetic potential of grain size. This study enhanced the understanding of cereal grain size variation and enabled the exploration of molecular breeding practices for the manipulation of this trait [60].

Seed vigour is a crucial trait in rice, affecting morphogenesis and yield improvement. A study by Guo et al. [42] identified QTLs for seed vigour using a germplasm collection and RIL population. Nine loci were identified through QTL mapping and six were selected as reliable. The authors identified 44 differentially expressed genes, with 44 being the most promising candidates associated with seed vigour. These findings contribute to the understanding of seed quality in rice production. Another important factor, i.e., structural variants (SVs), is crucial for the genetic control of essential traits in crops. A GWAS was performed on nine plant architecture traits (grain and biomass) to determine their genetic architecture and the association of allelic variants in gibberellin (GA) biosynthesis and signalling genes with these traits. A total of 101 SNP representative regions were associated with at least one of the nine traits, with GA candidate genes GA2ox5 and KS3 being significant markers affecting plant height and seed number [59].

Genome sequencing

Plant genomes contain crucial information, and sequencing tools have emerged to reveal it. Sanger sequencing (chain termination method) and Maxam–Gilbert chemical cleavage were among the first-generation sequencing methods that opened the way for the genomic era. When high-throughput sequencing isn't necessary, Sanger sequencing is beneficial in some cases, such as confirming plasmid constructs or PCR results. Nonetheless, the evolution of second-generation sequencing technology was motivated by the demand for low-cost but high-throughput information from vast plant genomes, which are divided into two types: sequencing by hybridization (SBH) and sequencing by synthesis (SBS). Illumina Tech, 454 Pyrosequencing and Ion Torrent are second-generation sequencing technologies. Short reads of 50 to 1000 bp are generated by first- and second-generation sequencing techniques, making them appropriate for focused sequencing of tiny amplicons, resequencing projects, and SNP calling.

Short-read sequencing is inadequate for large-scale projects, necessitating the adoption of third-generation platforms that integrate large-fragment single-molecule technologies. As an alternative, Pacific BioSciences (PacBio) invented single-molecule real-time (SMRT) sequencing to overcome many shortcomings of second-generation sequencing technology. Third-generation sequencing systems involve SMRT and Oxford Nanopore sequencing. It is feasible to use each short-read and long-read technologies for genome assembly. Short-read techniques, however, frequently result in incomplete assemblies (drafts), which result in missing sequencing, gene information loss, and lower precision downstream analysis [70]. Long reads produce overlapping sequences that enable (a) complete genome assemblies, (b) considerable structural changes to be circumvented and (c) the sequencing of extreme GC areas that would be impossible to sequence using a short-read sequencing technique. However, PacBio and Oxford Nanopore's long-read sequencing methods provide insufficient reads to cover some big, repetitive and complicated genomic areas.

High-throughput chromosome conformation capture (Hi-C) technologies and optical mapping can be employed to solve assembly constraints. Lieberman-Aiden et al. first described Hi-C in the year 2009 as a high-throughput genomic and epigenomic approach for capturing chromatin conformation (3C). In the Hi-C approach, the probability of contiguity of two loci in near proximity in 3-dimensional-nuclei space is greater than the two loci far apart. In *Triticum aestivum* and *Hordeum vulgare*, physical mapping data has been obtained using the Hi-C method and has been employed in genome assembly investigations [71]. 4-C and 5-C, i.e., chromosomal conformation capture on chip and chromosome conformation capture carbon copy techniques, respectively, were developed as a result of developments in methodology. Despite its low cost, Hi-C involves partial sequencing and results in mis-assemblies, making it vulnerable to errors. Unlike Hi-C, optical mapping is a technique for physically locating a specific enzyme or sequence motif using microscopy. For producing optical mappings, later technologies include DNA melting, competitive DNA binding, and enzymatic labelling. An optical map is bigger compared to reads acquired by short and long-read sequencing technologies, with an average molecular length of 225 kb. As a result, optical maps are being used to improve genome assembly, find massive SVs and phase haplotypes within genomic areas which are difficult to resolve using traditional DNA sequencing techniques [72]. Optical mapping has recently been used to refine the wheat genome assembly [73].

Transcriptomics

Genomics does not provide information on the fraction of the genome that is expressed or the amount of gene expression. Notably, the functional or regulatory proteins encoded by only about 1–2% of an organism's genome are expressed. Transcriptomics gives a plethora of information about the expressed genome by tracking the gene expression of an organism under various conditions, tissues (spatial transcriptome) and time periods (temporal transcriptome) [74, 75] (Table 2).

Techniques in transcriptomics

RNA transcripts were originally studied in the year 1970, when transcriptase silk moth mRNA libraries were reverse transcribed to cDNA [75]. Sanger sequencing was employed in the 1980s for sequencing RNA transcripts known as expressed sequence tags (ESTs) [101]. Later, numerous

Table 2 Studies on gene expression and transcriptome profiling in crops under biotic and abiotic stress

Crop	Tissue used	Method	Stress	Remarks	Reference
Oryza sativa	Leaves	Meta-expression analysis of microarray data	Cold	• Systematic analysis of transcriptome data was performed to identify the genes involved in cold stress responses and diurnal rhythmic patterns • Identified 885 and 572 cold-response genes that were up/downregulated at least twofold, respectively • The study hypothesized that there could be an interconnection between cold and circadian clock, achieved through the expression of *OsMAPK5*, *OsSnRK1a*, *OsHDA702*, and *OsPhyB*	[76]
	Leaves and shoot	RNA-seq	Adaptive and salinity	• Early H_2O_2-dependent signals are part of the coordinated activation of downstream genes that trigger a specific salt tolerance response • Salt-Responsive-ERF1 transcription factor (SERF1) was induced as early as 5 min in roots of tolerant variety with a peak at 30 min	[77]
	–	Transcriptome data, meta-analysis	Salt, submergence, heavy metal, and drought	• A comprehensive meta-analysis was performed on data mined from publically accessible transcriptomic abiotic stress (AbS) responsive datasets • The analysis identified unique and combined sets of 30 abiotic stress genes for further characterization regarding their functional role	[78]
Triticum aestivum	Shoots and roots	Microarray	Salinity	• Microarray analysis identified 1,811 salt-responsive genes with more than twofold expression in response to salt • Functional analysis of these genes should be done to understand the salt stress tolerance mechanism better	[79]
	Leaves	RNA-seq	Stripe rust and powdery mildew	• Transcriptome analysis of wheat line N9134, subjected to stripe rust and powdery mildew stress treatments, was performed • In the plant-pathogen interaction, more genes were activated in response to powdery mildew infection than stripe rust invasion	[80]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Zea mays	Tassels	RNA-seq	Drought	• RNA-Seq was performed using RNA of early developing tassel from 10 maize inbred lines under drought • Genes encoding enzymes involved in carbohydrate and lipid metabolism were significantly down-regulated in drought-stressed plants	[81]
	Leaves		Salinity	• Comparative transcriptome analysis between salt-sensitive and salt-tolerant maize lines identified differentially expressed transcription factors and suggested that WRKY TFs play an important role in salt tolerance • Snf1-related protein kinase2 (SnRK2) positively regulated ABA signaling and was selectively upregulated in the tolerant line	[82]
	Seedling leaves		Drought, salinity, heat and cold	• RNA-seq for global transcriptome profiling of seedling leaves identified 1,661, 2,019, 2,346, and 1,841 DEGs under salinity, drought, heat, and cold stress, respectively • Functional annotations indicated that the stress response involved hormone metabolism and signaling, transcription factors, very-long-chain fatty acid biosynthesis and lipid signaling	[83]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Hordeum vulgare L	Leaves and roots	Microarray	Drought	• Transcriptome analysis of root and leaf of CamB and Maresi genotypes under mild drought stress at the seedling revealed more changes in the transcriptome of roots than in the leaves • More differentially expressed genes (DEGs) are root-specific and are involved in primary root growth and the epigenetic control of chromatin and DNA methylation • Transcription factors from ERF, LOB, NAC, WRKY and bHLH families are important in the mild but not in the severe drought response of barley roots	[84]
	Roots	RNA-seq	Osmotic stress	• Suberin biosynthesis genes were upregulated in barley seminal roots exposed to polyethylene glycol 8000, resulting in endodermal suberization for reducing water loss through the apoplastic cell walls to the dry soil • By contrast, there was no or minimal effect on the expression of aquaporin genes	[85]
Sorghum bicolor L	Leaves	qRT-PCR and proteomics	Salinity	• Salt-responsive proteins were analyzed in G-46 and CSV 44F sorghum genotypes which showed that G-46 behaved as tolerant genotype performing better in terms of leaf function • The kafirin (seed storage protein) level of the two genotypes changed depending upon the concentration and exposure time to salts	[86]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Glycine max L. Merr	Seed coat	RNA-seq	Low rainfall, ozone and heat	• Abiotic stress-mediated gene expression analysis during the pod-filling stage discovered 49, 148 and 1,576 differentially expressed genes in the seed coat in response to drought, ozone and heat stress, respectively • Genes expressed in DNA replication and metabolic processes were enriched in the seed coat under heat stress as compared to drought and ozone	[87]
Camellia sinensis L	Leaves	qRT-PCR	Heat, cold, salt, ABA and drought	• Expression profiles of Homeodomain-leucine zipper (HD-Zip) family transcription factor genes (Cshdz) in tea plants were investigated under varied abiotic stress treatments, revealing a connection between stress resistance and *Cshdz* genes	[88]
Manihot esculenta L	Leaves and roots	RNA-seq	Drought and cold	• Genome-wide transcriptome analysis predicted 299 putative MeMYB genes in the cassava genome under drought and cold stresses, many of which exhibited different expression patterns in leaves • RNAi-driven repression of MeMYB2 genes resulted in tolerance against drought and cold	[89]
Cucurbita moschata L Duch	Leaves	RNA-seq	Powdery mildew	• Gene expression differences in powdery mildew treated and control plants of inbred line '112–2' identified six unigenes viz., bHLH87, ERF014, WRKY21, HSF, MLO3, and SGT1 as promising candidates involved in regulating the defense response	[90]
Lagenaria siceraria L	Leaves	RNA-seq	Powdery mildew	• RNA-Seq of leaf transcriptome of resistant and susceptible gourd inoculated with powdery mildew showed that genes of phenylpropanoid biosynthesis, starch and sucrose metabolism for cell wall cellulose synthesis were upregulated in disease-resistant plants	[91]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Gossypium herbaceum	Leaves	RNA-seq	Whitefly infestation	• RNA-Seq assessed transcriptomic differences between the resistant and susceptible cotton cultivars, revealing that WRKY40 and copper transport protein are hub genes regulating cotton defenses against whitefly	[92]
Nicotiana tabacum 'Petit Havana'	Leaves	Microarray	*Spodoptera litura* and *Helicoverpa armigera* infestation	• AtMYB12-expressing transgenic tobacco lines showed accumulation of the flavanol rutin and were resistant against *Spodoptera litura* and *Helicoverpa armigera* • *At*MYB12 regulated phenylpropanoid pathway in general and flavonol biosynthesis in particular	[93]
Vitis amurensis Rupr. cv. Zuoshan-1	Leaves	Solexa sequencing	Downy mildew (*Plasmopara viticola*)	• Solexa-based tag-sequencing proved a powerful tool for studying gene expression in *Plasmopara viticola* infected *Vitis amurensis* leaves and helped identify candidate genes and pathways involved in resistance	[94]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Solanum lycopersicum L	Leaves	RNA-seq	TYLCV and ToCV infection	• Differential gene expression using RNA-seq of TYLCV-resistant breeding line CLN2777A and susceptible breeding line TMXA48-4–0 revealed that resistance to TYLCV infection involves genes of cell wall reorganization, transcriptional regulation, defense response, ubiquitination, and metabolite synthesis	[95]
				• RNA-seq helped identify genes responsible for the development of symptoms of ToCV (chlorosis and anthocyanin accumulation) and TYLCV (yellowing, stunted growth, and leaf curl)	[96]
				• RNA-seq analysis of susceptible line Moneymaker and resistant line CLN2777A revealed that TYLCV infection induced the expression of genes involved in salicylic and jasmonic acid biosynthesis and the signal transduction of phytohormones, illustrating that phytohormones are essential for defending against TYLCV	[97]
Citrullus lanatus L	Root	RNA-seq	*Meloidogyne incognita* infestation	• RNA-seq analysis of the root transcript's abundance of resistant and susceptible watermelon varieties after *M. incognita* infection identified eight hundred thirt-five DEGs • Pathways of phenylpropane biosynthesis and carbon metabolism were significantly enriched in the resistant variety	[98]

Table 2 (continued)

Crop	Tissue used	Method	Stress	Remarks	Reference
Gossypium hirsutum L	Leaves	Microarray	ABA, cold, drought, salinity and alkalinity	• RNA-seq identified 3242, 1231, 3179, 480, and 3378 genes induced by ABA, cold, drought, salinity and pH stress, respectively • The WRKY family was implicated as a significant transcription factor family involved in the response to these stresses	[99]
	Root and shoot	qRT-PCR	Drought, dehydration and salt	• Expression profiling of cotton PIN genes showed that these are significantly upregulated by exogenous NAA and salicylic acid treatment, revealing their role in cotton root development and stress responses • The majority of cotton PIN genes contained auxin response elements (AuxREs) and salicylic acid (SA) responsive elements in their promoter regions	[100]

approaches for quantifying RNA transcripts were applied, including northern blotting and qRT-PCR [102]. On the other hand, these approaches only cover a portion of the transcriptome, not its entirety. SAGE, i.e., Serial Analysis of Gene Expression, was the first transcriptomics approach established and utilised in 1995 [103]. The SAGE approach builds a small sequence tag of length 10 to 14 bp to identify each transcript based on its unique position. These sequence tags are connected for formation of long serial molecules which may be cloned and sequenced. In order to assess a gene's expression, the total number of tags are quantified. The expression of a specific gene can be determined by quantifying the corresponding tag. SAGE can also assist in the discovery of new genes that are expressed in a particular tissue or under specific circumstances.

Microarrays, massively parallel signature sequencing (MPSS) and RNAseq are well-defined approaches offering high-throughput transcriptomics data. The hybridization of RNA transcripts to complementary probes mounted on a platform is utilised in microarrays to measure a set of RNA transcripts. It is employed to test thousands of genes at a reasonable cost per unit gene. The sensitivity and accuracy of this technology have been improved thanks to developments in array design and fluorescence detection devices. In a microarray, many probes are placed on a solid surface, such as glass or silicon. The fluorescently labelled transcripts hybridise with the probes on the chip, and the fluorescence intensity at each probe is used to quantify the transcript [104]. Nimble Gen invented a highly sophisticated high-density array employing mask-less photochemistry after the Affymetrix Gene chip array (Santa Clara, CA, USA) produced a high-density array first. Even though this technology is excellent for analysing transcripts in an organism, building probes for the chip needs prior information on ESTs and genome assembly.

Massive parallel signature sequencing (or MPSS) is a sequencing-based technique for quantitating mRNA transcripts in samples to analyse gene expression. To identify mRNA, MPSS looks for a 17–20 bp characteristic sequence near the 3′- end. Each distinctive sequence is initially transferred onto microbeads. This method guarantees that each microbead contains only one DNA sequence type. For sequencing and quantification, the microbeads are arrayed in a flow cell. Every signature sequence (MPSS tag) is compared to all other signatures in an MPSS dataset and all identical signatures are counted. To compute the expression level of a gene, the total number of signatures found in the samples is divided by the total number of signature sequences detected.

RNAseq is the technique of sequencing an organism's mRNA transcripts using next-generation sequencing (NGS) technologies. Sequencing costs have reduced dramatically, while accuracy has improved because of high-throughput sequencing technologies. Roche 454, Nanopore, Pac Bio, SOLiD and Illumina are examples of new sequencing technologies that have assisted the RNAseq technique in providing broad genome coverage. RNAseq gives a lot of information on the genes that are present, and their activation at specific times or under certain conditions [105]. As indicated by the number of articles published in the previous 10 years, NGS technologies have improved RNAseq over microarray in recent years.

In RNAseq, mRNA transcripts are isolated from other types of RNAs. Poly-A tail-specific probes are used to distinguish poly-A tail mRNAs from the rest of the RNA. Gel electrophoresis is used to exclude small RNAs based on their size. Hydrolysis or sonication are employed to fragment mRNAs to the read-length limit of sequencing technology. The desired mRNA is utilized to produce cDNA, which can be amplified if needed and then used as reads for NGS [106]. PacBio and Oxford Nanopore NGS systems sequence RNA directly without converting it to cDNA. It outperforms previous sequencing approaches by detecting altered nucleotides that would otherwise go undetected during cDNA synthesis and avoiding biases caused by cDNA amplification. The sensitivity and accuracy of RNAseq are dependent on the number of reads and genome coverage. RNAseq coverage of 70 × for common transcripts and 500 × for uncommon transcripts is recommended by the Encyclopaedia of DNA Elements (ENCODE).

NanoString is a hybridization-based approach which employs two probes for a target transcript: a biotin-labelled capture probe and a reporter probe (fluorescent barcode-labelled). The capture probe's biotin-avidin binding anchors the transcript to a solid surface, whilst the reporter probe's fluorescent barcode hybridises with a specific mRNA transcript. The immobilised mRNA transcripts are quantified using the NanoString nCounter analytical instrument. Because NanoString does not require processing, library preparation, or enzymes, it is superior to NGS-based techniques.

Transcriptomics: a key to understanding stress responses in plants

Transcriptome-level responses of plants to abiotic stress

Plants constantly alter their transcriptome to adapt to abiotic stress, thus forming a base for stress tolerance. Researchers have studied abiotic stress tolerance in plants, focusing on transcriptomic components to understand plant signalling responses. Li et al. [83] used RNA-seq to detect transcriptional changes in seedling maize leaves in response to multiple abiotic stresses. They identified 5330 DEGs between control and stress samples, revealing genes involved in hormone metabolism, signalling, TFs, VLCFA biosynthesis and lipid signalling. Rasmussen et al. [107] studied the response of *Arabidopsis thaliana* to combined abiotic stresses using microarray analysis. The results showed that transcripts exhibited varying responses to individual and combination stresses, 7% and 25%, respectively and were linked to plant defence. Moreover, 28% of the transcripts were involved in the maintenance of photosynthetic machinery. Zhu et al. [99] analyzed transcriptome dynamics in cotton under multiple abiotic stresses using microarray analysis. The study found functional genes and stress-related pathways, suggesting an interaction of responsive genes or pathways towards multiple abiotic stresses in cotton seedlings. Leisner et al. [87] found that soybean plants under multiple abiotic stresses (low rainfall, ozone and heat stress) experienced a decline in stomatal conductance and photosynthesis. RNAseq analysis of the seed coat identified 1576, 148 and 48 genes that were differentially expressed under heat, ozone, and drought stress, respectively. In another study, Muthuramalingam and co-workers [78] studied the responses of gene expression of rice to abiotic stresses using meta-analysis. They identified 1175 and 12,821 genes that were expressed meta-differentially and individually, respectively. Shen et al. [88] found that HD-Zip genes in tea plants were differentially upregulated in response to multiple abiotic stress conditions, viz., heat (5 genes), cold (6 genes), salt (9 genes), ABA (6 genes) and drought stress (3 genes). In another study, Kreszies et al. [85] examined the osmotic stress impact on barley root transcriptome using RNA-Seq approach, revealing the upregulation of genes linked to the suberin biosynthetic pathway. These data can be used to enhance abiotic stress tolerance by understanding differentially expressed genes and their role in signalling pathways. Ruan et al. [89] conducted a genome-wide transcriptome analysis in cassava, predicting 299 MYB gene family members. They found differential expression of MYB genes in leaves subjected to cold and drought conditions. Four MYB superfamily members responded to ABA treatment and additionally found that MeMYB2 acts as a negative regulator for drought and cold tolerance using RNAi technology. He et al. [100] evaluated the differential expression of 17 PIN efflux family members in stressed cotton plants, revealing salicylic acid and auxin responsive elements in their promoter region.

Transcriptome-level response of plants to biotic stress

Biological factors, viz., pathogen infestation and animal feeding, significantly impact plant growth and development. Plants can adjust gene expression and enzyme activity to induce signal induction and transmission in response to biotic stress. Moreover, understanding the relationship between transcriptome and biotic stress is crucial for investigating stress resistance. Plants are vulnerable to microbial pathogens and insect attacks, requiring various defence mechanisms to combat the invasion. For that, signalling molecules viz., SA, JA and ET play a significant role in plant-pathogen and plant–insect interactions. *A. thaliana* produces SA, JA and ET after attack by microbial pathogens and herbivores, with significant differences in quantity and time. Stress-related genes are overexpressed, and there may be an overlap between pathogens and insect-induced mutations in different attack patterns [108]. The cotton plant's transcriptional response to whitefly infestation involves genes encoding protein kinases, transcription factors, metabolite synthesis and phytohormone signalling. Furthermore, WRKY40 and transport protein are hub genes for defence, and it was reported that silencing GhMPK3 by virus-induced gene silencing (VIGS) resulted in suppression of MPK-WRKY-JA pathways, thus increasing whitefly infestation susceptibility [92]. Research showed that *A. thaliana* AtMYB12 expression in tobacco leads to increased phenylpropanoid pathway-related gene expression and flavonol accumulation. AtMYB12 regulates flavonol biosynthesis in transgenic tobacco lines, resulting in resistance to pests *S. litura* and *H. armigera* through increased Rutin accumulation [93]. A study analyzed 16 genes in pumpkins affected by powdery mildew, revealing six differential transcription levels. These genes, including bHLH87, WRKY21, ERF014, HSF, MLO3 and SGT1, were significantly upregulated or downregulated in varieties resistant to powdery mildew [90]. Deep transcriptome analysis of *Triticum aestivum* leaves inoculated with powdery mildew and stripe rust revealed that disease-resistant wheat cell lines utilized various defence mechanisms to enhance their resistance. The wheat line and pathogen share specific microbial genetic materials, which can help resist infection [80]. In another study, Zhang et al. [91] studied leaf transcriptome profiles of resistant and susceptible bottle gourd (*Lagenaria siceraria*) inoculated with powdery mildew. The study found that disease-resistant plants had upregulated expression levels of genes related to phenylpropanoid biosynthesis, starch, sucrose metabolism and plant-pathogen interaction pathways compared to susceptible varieties.

Transcriptome sequencing offers a large dataset for fruit plants also. Downy mildew, a global disease, significantly reduces grape yield and quality. Wu et al. [94] used a clone of *V. amurensis*, which exhibited cold hardiness and resistance to the disease. Gene expression levels were estimated using the Solexa sequencing method. Most unique tags (98.5%) showed similar expression levels, but 0.9% and 0.6% of unique tags increased or decreased more than five-fold in infected leaves. Tomato yellow leaf curl virus (TYLCV) is a critical disease affecting various plant species. Chen et al. [95] compared transcriptome sequencing data in resistant (R) and susceptible (S) tomato cultivars, finding differential expression patterns in 209 and 809 genes, respectively, among a total of 34,831 mapped transcripts. The study highlights the importance of up-regulated DEGs in R tomato lines, providing valuable resources for tomato research on resistance to TYLCV infection. Seo et al. analyzed TYLCV-infected tomato transcriptomes using RNA sequencing to analyze molecular mechanisms associated with symptoms like leaf curl, yellowing and stunting. They identified 1142 DEGs response to TYLCV infection, revealing significant changes in gene expression profiles likely linked to stunting and leaf-curling symptoms [96].

Recently, Song et al. [97] performed transcriptome profiling on TYLCV-resistant and susceptible lines to identify resistance mechanisms. They identified over 1000 DEGs related to metabolic processes, cellular processes, response to stimulus, biological regulation and signalling. The results showed that TYLCV infection induced the expression of genes involved in SA and JA biosynthesis and signal transduction of phytohormones, indicating that phytohormones are essential for tomatoes to defend against TYLCV. Zhu et al. [98] used a transcriptome dataset to study watermelon resistance to *M. incognita*, focusing on the resistant variety ‘Hongzi’ and the susceptible variety 'M16'. The study provided a theoretical basis for breeding resistant watermelon.

Proteomics

By giving a blueprint of likely gene products, advances in genomic technology have revolutionised the way we explore biological systems. Because of post-translational modifications, protein function, and localisation, the genome lacks a relation between mRNA and protein abundance. It doesn’t provide a biological snapshot of an organism at a specific stage of development. As a result, it’s vital to learn more about protein structure and interactions to understand how they affect plant growth and development. Proteomics is a high-throughput method for identifying and analysing protein expression in an organism's cell, tissue, or organelle at a specific time and a specific circumstance [109] (Table 3). The initial report on two-dimensional electrophoresis (2-DE) was published in 1975, providing the first peek into cell protein levels and isoforms. At the opening of the 2-DE symposium in Siena, Italy, in 1994, Marc Wilkins coined the term “proteomics” as an extension of the word "proteome" (PROTein complement of the genOME) [110]. Proteome profiling can yield a lot of information about distinct metabolic processes in a biological system and their interactions with different regulatory pathways. Proteomics is a powerful tool that delivers a more accurate and reliable picture of cell function than genomics.

Table 3 Use of different proteomics approaches for investigating crop responsiveness to biotic and abiotic stresses

Crop	Stress	Methodology	Remarks	References
Oryza sativa L	Drought	LC–MS and MS	• The ClpD1 protease was upregulated in mature leaf laminae under drought stress, while porphyrin and chlorophyll biosynthesis pathways were downregulated	[111]
	Bakanae disease	TMT7MS	• 6-plex tandem mass tag approach used for relative quantitative proteomic comparison of infected and uninfected rice seedlings in resistant versus susceptible genotype showed that the aquaporin protein PIP2–2 was sharply upregulated and might be involved in pathogen defense against bakanae disease	[112]
	Salinity	Nano-LCMS/MS	• Proteome profiling of salt-sensitive and salt-tolerant cultivars revealed relatively higher abundance of proteins associated with photosynthesis, energy metabolism, glutathione metabolism, nitrogen metabolism, and stress defenses in the salt-tolerant cultivar	[113]
	Salinity	iTRAQ	• iTRAQ-based identification of the differentially expressed proteins in two genotypes of contrasting salt tolerance showed that the numbers of identified proteins are in direct proportion to the increasing salt stress levels, and that rice cell membrane integrity was inversely correlated while root activity was positively correlated with the salt concentration	[114]
Triticum aestivum L	Drought	2D-PAGE	• Proteomic analysis of the drought-tolerant cultivar Hanxuan 10 and drought-sensitive Chinese Spring showed that DEPs related to stress defense were upregulated in tolerant than in sensitive • Phosphorylation levels of HSP60, HSP90, and HOP were upregulated in tolerant cultivar under drought stress	[115]
	Yellow rust	nanoLC ESI–MS/MS	• The study on differentially regulated early response proteins in wheat leaves infected by Puccinia striiformis f. sp. tritici isolates showed that pathogen related protein 1 and 4 (PR1, PR4), Glutathione S transferase (GST) are vital for systemic acquired resistance	[116]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
	Osmotic stress	2DE, MALDI–TOF–TOF–MS	• The response of calli to polyethylene glycol (PEG6000) was studied using diagonal two-dimensional electrophoresis (D-2DE) combined with mass spectrometry, and the proteins identified through this technique provide new insights into how calli respond to osmotic stress	[117]
	Stripe rust	iTRAQ	• The quantitative proteomic comparison of the incompatible Puccinia striiformis f. sp. tritici race CYR23 in infected and non-infected leaves of the wheat cultivar Suwon11 showed that 530 proteins were differentially expressed including reactive oxygen species (ROS) metabolism-related proteins, peptidyl–prolyl cis–trans isomerases (PPIases), RNA-binding proteins (RBPs), and chaperonins	[118]
Zea mays L	Drought	iTRAQ and LC–MS/MS	• Comparative analysis of protein expression in the seedling roots of the drought tolerant Chang 7–2 and sensitive TS141 varieties under 20% polyethylene glycol 6000 (PEG 6000) revealed the mechanism of tolerance by strengthening the cell wall through the up-regulation of xyloglucan endotransglucosylase/hydrolase • Acyl-CoA-binding protein, lipid binding protein, and asparagine synthetase relieve the extravasation of cellular contents, maintain osmotic balance, and stabilize the membrane system of tolerant variety	[119]
	Drought	iTRAQ and LC–MS/MS	• The protein expression profiles of inbred lines YE8112 and MO17 in developing kernels against drought stress revealed that YE8112 kernels were drought tolerant because of their enhanced redox PTMs and epigenetic regulation, enhanced HSPs expression, enriched CHO metabolism, and upregulated expression of seed storage proteins	[120]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
	Drought	iTRAQ	• Comparative analysis of two maize inbred lines at the seedling stage showed that the tolerant variety YE8112 had significantly enriched DAPs associated with the photosynthesis antenna proteins pathway, whilst those in the susceptible MO17 were related to C5-branched dibasic acid metabolism and RNA transport pathways	[121]
	Maize chlorotic mottle virus	iTRAQ	• Proteomic analysis performed on maize cv. B73 revealed that two DAPs, viz., ZmPDIL-1 and ZmPrx5 enhance host susceptibility to MCMV infection	[122]
	Northern corn leaf blight	2DE and MS	• The comparative proteomic study underlying the defense responses of the maize resistant line A619 Ht2 to *S. turcica* race 13 revealed that resistance processes of A619 Ht2 mainly resided on directly releasing defense proteins, and the modulation of primary metabolism related to photosynthesis	[123]
	Downy mildew	2D-PAGE, MLADI–TOF/MS, MASCOT	• The study between the mock-inoculated and sorghum downy mildew-inoculated leaves in the susceptible genotypes of maize (UMI79 and CM500) revealed that most peptide mass fingerprints were that of lipoxygenase, DEAD-box ATP-dependent RNA helicases, microtubule-associated protein and disulphide isomerase	[124]
Hordeum vulgare L	Fusarium head blight	2D-PAGE, MS	• Eleven differentially expressed proteins were detected in response to fungal infection and three were connected with the growing location of the plants • The proteins are involved in cell functions of transcriptional regulation, defence response, nutrient reservoirs and starch biosynthesis	[125]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
	Salinity	2-DE, Mass spectrometry	• Tolerant near-isogenic lines N33 and T46, differing in QTL for salinity tolerance on chromosome 2H, were used for proteomic studies and the higher activities of antioxidant enzymes were detected under the background of salt tolerance QTL *QSl.TxNn.2H* • Proteins associated with photosynthesis, reactive oxygen species scavenging, and ATP synthase were explicitly upregulated in the tolerant NILs	[126]
	Waterlogging stress	TMS	• Proteomic study on two barley genotypes with contrasting sensitivity to waterlogging stress revealed that the tolerant genotypes produce more ethylene, scavenge more ROS and undergo more photosynthesis than the sensitive genotypes	[126]
Sorghum bicolor L	Heavy metal toxicity	2D-PAGE	• Cadmium-induced morpho-physiological alterations in *Sorghum bicolor* leaves were studied • There were significant changes in carbohydrate metabolism, transcriptional regulation and translation as cadmium stress inhibited carbon fixation, ATP production and protein synthesis	[127]
	Salinity	SDS-PAGE and Immunoblotting assay	• Salt-responsive proteins in sorghum genotypes G-46 and CSV 44F showed that the two genotypes adopted distinct approaches towards salinity, and G-46 performed better with respect to leaf function	[86]
	Chilo partellus	LC–MS/MS	• Upon infestation with insect pest *Chilo partellus* sorghum genotypes responded by suppressing protein and amino acid biosynthesis, and inducing proteins involved in maintaining photosynthesis and responding to stresses • The resistant genotypes displayed two-fold higher numbers of unique proteins than the susceptible genotype, most of which were involved in catalytic activities	[128]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
Cicer arietinum L	Salinity	2-DE and LC–MS/MS	• Sixty-five differential expressed proteins were classified based on their putative functions in two chickpea genotypes: salt-tolerant and susceptible • The salt tolerant was enriched in proteins related to photosynthesis (39%), stress response (21%) and protein synthesis/degradation (14%)	[129]
Glycine Max L	Water and heat	2-DE and MALDI-TOF MS	• Proteomic analysis to assess the effect of water stress, heat stress, and combined stresses on the regulation of leaf proteins in two contrasting soybean cultivars identified a candidate protein MED37C as a probable mediator of RNA polymerase transcription II associated with heat shock proteins and can improve plant tolerance against multiple stresses	[130]
	Flooding and salinity	MALDI-TOF/TOF MS	• Proteomic analysis of root and hypocotyl in seedlings grown under combined stress of flooding and high salinity detected enzymes not previously reported to be associated with either flooding or salt stress, e.g. 6-phosphogluconate dehydrogenase, dihydroflavonol 4-reductase, flavonol 4′-sulfotransferase, arginase	[131]
Solanum lycopersicum L	Cucumber mosaic virus (CMV)	2-DE, DIGE and nLC-ESI-IT-MS/MS	• The majority of the DEPs in the leaf proteome expressed during plant-virus interaction in wild-type and transgenic tomato were related to photosynthesis (38%), primary metabolism (18%), and defense activity (14%) and were actively downregulated by CMV in infected leaves	[132]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
	TYLCV infection	2-DE and MALDI-TOF-TOF MS	• Proteomic approach in combination with qRT PCR was used to investigate the molecular mechanism of tomato leaf defense against TYLCV infection • Eighty-six DEPs were identified and a putative interaction network between tomato leaves and TYLCV infection was developed • Interestingly, protein expression levels and qRT-PCR analyses showed opposite patterns, with 2-DE showing down-regulation and qRT-PCR showing up-regulation, indicating that protein formation involves complicated translation and modification	[133]
Vitis vinifera L	Downy mildew	2-DE and Mass spectrometry	• Comparative proteomic analysis of leaves from the susceptible cultivar Pinot Noir showed that the inoculation with *P. viticola* resulted in the expression of eighty-two unique differentially expressed grapevine proteins • There was an early modulation of about fifty proteins in the host. However, with time of colonization, the number reduced to 18, most of which were isoforms of different PR-10 pathogenesis-related proteins	[134]
Vigna unguiculata L	Combined nematode and drought	LC–MS/MS	• Proteins involved in activating the antioxidant mechanisms, production of NB-LRR proteins and secondary metabolites were identified in cowpea when subjected to nematode and drought stresses individually and combined	[135]
Brassica campestris L	High and low temperature	TMT and LC–MS/MS	• This study reported redox homeostasis, carbohydrate metabolism, heat-shock proteins, chaperones and signal transduction pathways to be associated with tolerance to temperature stress in cultivar Wucai	[136]
Abelmoschus esculentus L	Salinity	TMT and LC–MS/MS	• TMT label-based quantitative proteomics identified DEPs under NaCl treatment in okra, associated with protein processing in endoplasmic reticulum and heat shock proteins	[137]

Table 3 (continued)

Crop	Stress	Methodology	Remarks	References
Camellia sinensis L	Cold	iTRAQ	• The study investigated the role of γ-aminobutyric acid (GABA) in tea plants, revealing that GABA could play a role in carbon and nitrogen metabolism under cold stress	[138]

Proteomics techniques

Plants have lately been subjected to several proteomics approaches that have recently been developed and implemented. These advancements created an opportunity for high-throughput proteome analysis, allowing scientists to identify and localise protein–protein interactions and post-translational modifications (PTMs). Identification/quantification, extraction and separation (gel-based and gel-free) are the three fundamental steps in most proteomics methodologies [139]. Gel-free processes include label-free techniques like LC–MS and tag-based techniques like ICAT and iTRAQ. Due to the complexity and dynamic nature of the plant proteome, a single method cannot accurately evaluate it. As a result, numerous techniques are employed to improve plant proteome knowledge, resolution, and coverage. The proteome's study methodology is determined by numerous factors, which include the availability of resources, type of facilities available and applications, viz., global or targeted profiling [140]

Proteome profiling on a global scale

Global proteome profiling is one of the finest methods for comparing two or more proteomes or making a reference proteome map [141]. Gel-free proteomics is becoming more common as years roll on. Compared to gel-based proteomics, it has superior reproducibility and less bias [142].

Gel-based methodologies

These strategies are the most popular, adaptable and recognized protein separation and quantification techniques. They are less costly than gel-free approaches and may be used to broadly characterise protein isoforms and identify low-abundance proteins. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the workhorse of proteomics due to its accessibility and familiarity. It is often used in expression proteomics research. Isoelectric point (pI) and molecular mass (M) are used to distinguish proteins. Proteins are split into two groups based on their molecular mass (M) and whether or not they contain 2-mercaptoethanol. The proteins may be seen using staining dyes like Coomassie blue, silver nitrate or SYPRO Ruby. Difference in-gel electrophoresis (DIGE) aims to overcome the drawbacks of 2D-PAGE, including gel-to-gel variations and inadequate repeatability. A significant number of protein samples have been fluorescently labelled at their lysine residues with different fluorophores (CyDye2, CyDye5, CyDye3) and Coomassie Blue, silver nitrate, or SYPRO Ruby, may be separated using this method on a single gel. Protein expression variations in response to biotic and abiotic stresses may be assessed using the DIGE technique [143]. To reduce co-migration interferences from 2D-PAGE, three-dimensional gel electrophoresis (3DGE) is used [144]. It accurately identifies proteins and PTMs using two separate buffers with different ion carriers [145].

After digestion has extracted peptides, proteins of interest are identified using mass spectrometry (MS) [146]. Various computer approaches to aid protein identification based on peptide mass and fragmentation (MS/MS) data. There are three phases in the MS protein identification process. Turning molecules into gas-phase ions, separating them in an electric or magnetic field depending on their mass-to-charge ratio (m/z) and identifying separated ions with a certain m/z value. Some of the ionisation methods employed include electrospray ionisation (ESI), matrix-assisted laser desorption ionisation (MALDI), and surface-enhanced laser desorption/ionization (SELDI) [147].

Gel-free methodologies

The limitations of gel-based techniques, such as the inability to segregate the entire proteome, the difficulty in detecting low-abundance proteins and the length of the procedure, are being addressed by gel-free alternatives. Quantitative techniques include tag-based labelling (ICAT, iTRAQ), metabolic labelling (SILAC) and label-free (MudPIT).

Isotope-coded affinity tagging (ICAT) is a protein quantification method that utilizes a biotin affinity tag, a stable isotope linker and a reactive component that latches onto protein thiol groups (cysteines) in vitro. Chromatography is used to separate the tagged tryptic peptides before mass spectrometry (MS) is used to detect them [148]. ICAT is largely used to aid in the discovery of novel proteins that affect a specific cultivar's important biological function [149].

Using isobaric tags, the N-terminus and side-chain amine groups of proteins are identified in the multiplex protein quantification method known as iTRAQ (isobaric tagging for relative and absolute quantification). Protein quantification from several sources in a single test has much higher sensitivity than ICAT [150]. Crop breeders utilise this technique to detect biotic and abiotic stress markers, which can then be used to develop genetically modified crops [151].

The most effective metabolic labelling tool for dynamic quantitative plant proteome research is stable isotope labeling by amino acid in cell culture (SILAC). It employs in-vivo labelling of cell populations cultivated in N14 or N15-containing medium [152]. Identifying proteome alterations in signalling pathways generated by PTMs in response to stress is valuable [153].

MudPIT is a shotgun proteomics approach for analysing difficult multi-dimensional proteins [154]. It is a less complicated and more sensitive technology for detecting low-abundance proteins. This method separates digested proteins using biphasic or triphasic microcapillary columns, which are then analysed using tandem MS. This technology has been utilised to explore the mechanisms regulating the number of rice tillers [155].

Targeted proteome profiling

It describes the proteome analysis of interacting proteins or post-transcriptionally changed proteins using specific PTM stains, antibodies or targeted MS techniques [156]. Its three types are gel-based targeted proteomics, affinity and reactive chemistry-based targeted proteomics and MS-based targeted proteomics.

Gel-based proteomics

2D-PAGE is used to analyse the global proteome, which is then labelled with phosphoprotein-specific gel dye (Pro-Q Diamond; PTM specific stain). These techniques are no longer employed since it is difficult to identify less common proteins [157].

Proteomics based on affinity and reactive chemistry

Some of the techniques used to identify, enrich, and purify specific proteins include immunoprecipitation (IP), immobilised metal affinity chromatography (IMAC), strong anion exchange (SAX) and strong cation exchange (SCX). These methods can be used on their own or in combination to increase efficiency [158]

Proteomics based on mass spectrometry

The techniques operate by identifying signals produced by ion transitions during fragmentation using a mass spectrometer. Additional tools employed include tandem MS, linear trap quadrupole orbitrap (LTQ-Orbitrap), quadrupole trap (Q-trap) and triple quadrupole. Multiple reaction monitoring (MRM) is the process of recognizing multiple changes, whereas selected reaction monitoring (SRM) is the process of identifying transitions in a triple quadrupole [159]. The approaches mentioned above, on the other hand, have concerns with sample accuracy. To overcome this problem SRM/MRM methods are isotopically labelled.

Proteomics bioinformatics

A vast amount of high-quality protein-expression data may now be produced because of advances in proteomics technology. Coupling this data with other omics technologies, including genomes, transcriptomics, metabolomics and phenomics is challenging. Bioinformatics tools are crucial in removing this barrier since they reduce analysis time and produce substantial statistical results. Proteomics identification database (PRIDE) is one of the most widely utilised proteomics databases [160], peptide atlas [161], and mass spectrometry interactive virtual environment (MassIVE). Plant proteomics database (PPDB), 1001 Proteomes, Pep2 Pro Database, DIPOS and others are some of the resources available. Many extensive databases for plant proteomics have been produced [162], and various web-based prediction tools such as GelMap [163], MRMaid [164], Peptide atlas SRM experiment library (PASSEL) [165] have been created to aid in the investigation of proteomes.

Proteomic study of plants in response to stress

Proteins are essential functional entities in plant stress response, enabling understanding of protein networks and metabolic pathways. Identifying a master regulator protein crucial for abiotic and biotic stress response is crucial for genetically engineered crop development and modelling. This helps to understand stress response and promotes better crop adaptation.

Proteome-level response of plants to abiotic stress

Katam et al. [130] examined the impact of multiple abiotic stresses on leaf protein regulation in soybean cultivars. The study provided insights into proteome and enzyme responses towards multiple stresses in soybean under field conditions. Das et al. [166] examined differential protein expression in soybean (*Glycine max* L.) for drought and heat stress responses. They found that 44 abiotic stress-responsive proteins affected signalling cascades and molecular processes and many differentially expressed photosynthesis-related proteins impacted RuBisCO regulation, electron transport and Calvin cycle during abiotic stress.

A study by Alam et al. [131] using MALDI-TOF/TOF mass spectrometry identified 43 responsive proteins in salt-stressed soybean, with 29 up-regulated, 8 down-regulated and 6 novel proteins under salinity stress. ITRAQ and LC–MS/MS were used to analyze protein expression in maize seedling roots of drought-tolerant and sensitive varieties. Seven thousand seven hundred twenty-three differentially expressed proteins (DEPs) were identified. The results showed that DEPs in tolerant varieties were linked to ribosome pathways, glycolysis/gluconeogenesis, amino sugar metabolism, while in the susceptible DEPs were associated with metabolic pathways, phenylpropanoid biosynthesis, and starch/sucrose metabolism [119]. Zenda et al. [121] used an iTRAQ-based quantitative strategy to study maize drought stress responses at the protein level. They analyzed 721 differentially abundant proteins (DAPs) in two maize inbred lines, revealing common and unique proteins accumulated in response to water limitation in maize. Wang et al. [120] analyzed proteomics of filling-kernel proteomes of two drought-tolerant maize lines, revealing diverse molecular actors mediating drought tolerance.

A study by Punia et al. [86] analyzed salt-stress responsive protein expression patterns in G-46 and CSV 44F sorghum genotypes based on their salinity tolerance potential. Results showed that G-46 performed better in leaf function, and Hsp 70 expression was within the physiological range, thus behaving as a tolerant genotype under salinity. Goche et al. [167] identified complex protein networks that underpin sorghum responses to drought, offering new targets for investigating drought tolerance in sorghum. A nano-LCMS/MS proteome profile was used to study salt tolerance in sensitive and tolerant rice cultivars viz., Jhelum and Vytilla-4. Results showed 770 and 514 protein spots in the salt-sensitive and tolerant cultivars, respectively. DEPs were found to be linked with major metabolic pathways, viz., photosynthesis, energy metabolism, amino acid metabolism, nitrogen assimilation, stress and signalling pathways [113]. A comparative proteomics study in two contrasting rice genotypes using iTRAQ identified 368 and 491 proteins up-regulated in tolerant rice genotype LYP9 under moderate and high salinity stress, respectively, at the tillering stage [114].

By performing biochemical and proteomic analysis in two pairs of Barley (*Hordeum vulgare L.*) near-isogenic lines (NILs) under salinity stress, Zhu et al. [126] identified 53 and 51 differentially expressed proteins in leaves and roots, respectively. A study analyzed two barley genotypes under waterlogging stress, TF57 (sensitive) and TF58 (tolerant). Differentially expressed proteins were identified in leaves, adventitious roots, nodal roots, and seminal roots, suggesting they could be biomarkers for improving waterlogging tolerance in barley [168]. Yuan et al. [136] used tandem mass tag and LC–MS/MS to identify differentially expressed proteins in Wucai (*Brassica campestris* L) treated with low and high-temperature stress. The study identified a total of 172 proteins upregulated in response to both stress levels. Zhan et al. [137] conducted a comparative proteomic analysis (TMT labelling and LC–MS/MS) of Okra seedlings grown in 0 or 300 mmol L^{-1} NaCl treatment to understand the effect of salt stress on protein levels. The study identified 317 differentially expressed proteins, with 165 upregulated and 152 downregulated under salt-stress conditions. These results offer valuable insights into candidate proteins and molecular mechanisms involved in okra plants' response to salt stress. A study aimed to isolate novel salt-stress-responsive proteins in chickpea leaves using two contrasting genotypes. 65 DEPs were characterized using 2-DE and LC–MS/MS analyses. Comparative proteomic analyses revealed some unexpected proteins not previously reported in chickpea or related to salinity [129]. Zhu et al. [138] studied GABA tolerance mechanisms in tea plants under optimum and low temperatures by analyzing physiological and iTRAQ-based proteomic data. They found GABA may contribute to carbon and nitrogen metabolism under cold stress.

Proteome-level response of plants to biotic stress

Proteomics profile analysis characterized 186 proteins in tomatoes infected with *B. cinerea*, revealing their role in defence against the disease [169]. Di et al. analyzed the plant-virus interaction in wild-type and transgenic tomatoes using proteomic analysis. Out of 2084 protein spots, 50 DEPs were detected using 2D gel electrophoresis, mainly related to photosynthesis, defence activity, and primary metabolism [132]. Huang et al. [133] utilized a proteomic approach to analyze the interaction between resistant/susceptible tomato cultivars and TYLCV infection. The study identified 86 differentially expressed proteins responsible for photosynthesis, proteometabolism, carbohydrate metabolism, signal transduction, chaperones, detoxification, antioxidation and amino acid metabolism. Milli et al. [134] analyzed proteomes of grapevine leaves infected with *P. viticola*, identifying 82 differentially expressed proteins, with most resistance-related proteins belonging to the PR-10 group. In another study, a comparative iTRAQ proteomics analysis of wheat stripe rust resistance in Suwon11 cultivar revealed ROS metabolism-related proteins, including PPIases, RBPs and chaperonins, involved in the response to Pst infection [118].

A comparative proteomic analysis of three sorghum genotypes with variable resistance to spotted stem borer (*Chilo partellus*) revealed putative leaf *C. partellus* responsive proteins. These proteins were significantly up-regulated in resistant sorghum genotypes upon pest infestation, affecting stress and defense, photosynthesis, small molecule biosynthesis, amino acid metabolism, catalytic and translation regulation activities [128]. Ribeiro et al. [135] analyzed the protein profile of cowpea (*Vigna unguiculata* L. Walp.) under biotic and abiotic stresses. Shotgun proteomics revealed 2345 proteins, with 1040 differentially abundant. These proteins are involved in essential biological processes like transcription and cell signalling. These differentially abundant proteins help understand commonalities and differences in cowpea responses to single and combined stresses.

Dang et al. [122] conducted iTRAQ-based comparative proteomic analysis on maize infected with MCMV. They identified 972 DAPs, with 661 proteins with increased and 311 with reduced abundance. Functional annotations and photosynthetic activity measurements showed decreased photosynthesis and significant changes in ribosomal proteins, stress responses, oxidation–reduction and redox homeostasis. Zhang et al. [123] studied the molecular mechanisms underlying defence responses of maize resistant line A619 Ht2 to *S. turcica* using 2-DE and MS. The study found a complex regulatory network in the interaction between *S. turcia* and A619 Ht2, mainly releasing defence proteins, modulating primary metabolism, and affecting photosynthesis and carbohydrate metabolism.

Phenomics

Genomics has revolutionised plant breeding in the recent two decades due to lower genotyping costs and the use of novel methods such as linkage mapping, genome-wide selection, genome-wide association studies and rapid generation advances. Plant phenomics, on the other hand, is a relatively new science that uses techniques and approaches to study plant growth, development, performance and composition. As a result, it has fallen behind genomics in terms of progress [170]. Plant phenotyping is a fast-increasing discipline for accurate genotyping as it is required for precision genetic mapping and genome-wide selection. For instance, a comparative mQTL mapping between rice and maize covers from high-throughput field phenotyping to cellular-level imaging. Conventional field phenotyping is labour-intensive, expensive, and subjective. However, interest in field-based high-throughput phenotyping (HTP) has increased during the past decade, particularly for predicting physiological and agronomic features [171]. HTP has demonstrated its potential for non-destructive phenotyping of agronomic, physiological and stress-related traits by utilizing high throughput techniques and platforms, image processing, applying algorithms to extract raw data and connecting the processed data to the target traits [172]. For improved precision, throughput and accuracy in monitoring different plant characteristics at various developmental stages, new aerial or ground-based HTP platforms have been developed.

Phenotyping platforms for plants

The image sensor being employed affects HTP. By combining mobility, speed and built-in data storage, modern phenotyping devices have enhanced data-capture capabilities at a relatively cheap cost. Due to flying regulations, certain nations have not yet adopted unmanned aerial vehicles (UAVs), despite their high acceptance rate, cheap cost and technological needs. Numerous carts and tractor-mounted equipment have been used for various crops, but their usage depends on the stage. Furthermore, various low-cost mobile devices can obtain spectral and time-series data. On the other hand, these handheld devices face consistency and throughput issues and they give less canopy coverage because they are typically positioned above poles.

In both controlled and field environments, imaging sensors have enabled capturing high-resolution, multidimensional data from plants to analyse plant growth, yield, stress and physiological processes [173]. Plant scientists now have access to a wealth of data due to the recent invention of sensor technology that measures gamma-ray reflection to radio wave sections of the electromagnetic spectrum. Some of the techniques have been discussed in the following sections:

Imaging in RGB and visible light In the visible part of the electromagnetic spectrum, RGB, conventional, and digital cameras capture accurate colour images. RGB is the cheapest and most commonly used sensor for plant phenotyping investigations. These sensors reflect red, green, and blue colours from the visible spectrum. This technique has been used to quantify plant biomass, different pigments, tiller count, yield attributes, blooming duration, biotic stressors, plant height, germination rates, and emergence rates.

Imaging with multiple spectral bands Multispectral cameras collect data on specific wavelength bands in the visible and infrared sections of the spectrum [174]. The vegetation bands that offer information on photosynthetic efficiency, pigments, nutritional state, water status and plant senescence are extracted using these reflection bands. The most significant metrics utilized are the simple ratio (SR), water index (WI), anthocyanin reflection index (ARI) and normalized differentiation vegetation index (NDVI).

Hyperspectral imaging (HSI) It is a type of imaging that, due to the sensor's short bandwidth, these image sensors cover the whole visible and infrared regions with a high spatial resolution by covering reflection from all areas. These sensors offer the highest spatial and spectral resolution, providing more valuable data. Plant health, leaf growth, grain yield prediction, biotic stressors, water status, plant height, and chlorophyll content have all been studied using this imaging technology [175].

Thermal imaging These sensors provide information about plant water status by sensing infrared reflection and determining canopy temperature and transpiration rate [176]. Plant water status, disease-infected plants, and kernel maturity have all been detected via thermal imaging [177].

Fluorescence imaging By recording photosystem II's fluorescence emissions, fluorescence sensors provide information regarding photochemistry changes. Plants absorb a certain part of the electromagnetic spectrum and emit a distinct spectrum of light. Fluorescence sensors measure the rate of photosynthesis, the amount of chlorophyll in the plant, and other physiological activities [178].

X-ray computed tomography These imaging sensors help create 3D tomographic images of things from a large number of 2D radiographic images captured using computer-processed X-rays. By separating objects based on their densities, images provide root structures. Root traits, tiller morphology, and grain quality have all been studied using X-ray CT [179]. There are various other imaging sensors, including positron-emission tomography, magnetic resonance imaging, SONAR, laser scanning, LIDAR and flight time, in addition to all of the above.

Phenomics for crop improvement

Precise phenotyping accurately maps genes and QTLs for specific traits, enabling forward and reverse phenomics applications for genetic improvement in crop plants. Automated high-through put imaging technologies can detect the best germplasm lines through non-invasive methods like colour imaging, far infrared imaging, lidar and magnetic resonance imaging. These non-invasive approaches enable rapid phenotyping of traits, allowing for rapid growth parameter measurement and analysis of root systems in crops [180].

Root systems can also be imaged in laboratories and greenhouses without damaging plant samples using techniques like electrical resistance tomography, electrical capacitance, X-ray computed tomography and positron emission tomography [181]. For example, RGB imaging estimates canopy and root systems using visible light phenotyping [182]. Different studies have utilized visible light imaging techniques in RGB setups to determine crop phenotyping parameters, such as analyzing root systems in *Triticum durum* grown in soil-filled rhizoboxes [183], while RGB colour-based imaging has also been used to assess plant pathosystem and disease symptoms [184].

The role of stomatal conductance under salinity stress has been confirmed in young barley and wheat seedlings using infrared thermography [185]. Chlorophyll fluorescence imaging has been used to screen for abiotic stress responses in tobacco, canola, and cotton crops using pulse-amplitude-modulated (PAM) instruments [186]. Similar methods have been used to detect and quantify disease symptoms in barley crops [187]. Jansen et al. [188] used chlorophyll fluorescence and 2D digital imaging of plant growth to monitor plant reactions in Arabidopsis under drought and chilling stress. Rungrat et al. [189] examined chlorophyll fluorescence for monitoring photosynthetic activity in *Arabidopsis thaliana* under abiotic stresses. Qiu et al. [190] found significant differences in leaf, air and canopy temperature in melons, tomatoes, and lettuce under drought and high temperature stress using infrared imaging. The study proposed using the transpiration transfer coefficient (hat) to detect plant environmental stresses. In another study, Wedeking et al. [191] utilized IRT to monitor leaf temperature and transpiration in *Beta vulgaris* plants under drought stress. Overall, phenomics applications have been used voguely to evaluate the phenotypic parameters in crops under abiotic stress studies, and there is an increasing trend in using it for biotic stress conditions too.

Challenges and prospects of omics approaches

Emerging genomics approaches

Whole genome sequencing (WGS) initiatives have been impeded by large and repetitive cereal genome sequences and a lack of modern technologies and algorithms capable of producing and assembling large and accurate sequences [192]. It is possibly the most important area where extensive international collaborations are needed. Technology developments would simplify future large-scale and reference-quality genome assemblies because the cost-to-outcome difference is minimal, even though large-scale genome sequence generation and assembly are still expensive. It is realistic to anticipate that the difference between whole-genome genotyping and WGS will be insignificant as genome assembly advances [193]. As sequencing and computational capabilities improve, productivity per input unit will increase, benefiting cereal geneticists and breeders. Advances will aid the quick and precise mapping of traits in genomics. Furthermore, as more dense marker data becomes accessible, methods for predicting genotypic or breeding value will improve, allowing for greater genetic gains per unit of expense and time.

Epigenomics is another area which has huge prospects. It is a fusion of epigenetics and genomics and aims to understand genetic regulation and its impact on cellular growth and stress responses. The bisulfite sequencing technique can identify DNA methylation status in the genome and it has been validated in many crops, including tomato, maize and soybean crops [194]. Methylation-sensitive amplified polymorphism (MSAP) quantification is common, and it has been used in wheat and foxtail millet crops under salinity stress [195]. Chrinin immunoprecipitation sequencing (ChIP-Seq) is a unique approach for analyzing histone proteins and DNA methylation, and it has been used in rice crop under drought stress. These data suggest that epigenomics applications could significantly contribute to crop improvement in response to environmental stresses.

There is a novel approach to understand a specie's complete genome make-up known as pangenomics. It is the complete genomic makeup of a species, consisting of core and dispensable genes. Core genes are shared by all individuals, while dispensable genes are individual-specific and present in some individuals but not all [196, 197]. Studies have shown that dispensable genes are crucial for maintaining crop diversity and improving quality. Pangenomics can be used to improve crops, as multiple crop wild relatives (CWRs) have been used in breeding programs for their quantitative and adaptive traits. These traits, such as yield and grain size, have been subjected to intensive selection during domestication and breeding improvement in crops like rice, sorghum and wheat. Thus, pangenomic studies could help mine elite genes in CWRs for crop improvement.

Single-cell genomic analysis is an innovative upcoming approach to studying cells involved in tissue development, regeneration and repair [198]. The approach can be extended to study biological diversity in plant cells for understanding organ development and environmental stress response. There are challenges in single-cell preparation, DNA/RNA amplification, sequencing, and bioinformatics analysis, but the rapid evolution of single-cell technologies will address these issues in the near future.

Advances in transcriptomics technologies

Transcriptomic studies have experienced many obstacles in the past, most of which were resolved as the techniques advanced. The microarray has the limitation that it can only be used to assess the expression level of 'known' genes. This limitation was overcome by RNAseq, which provides a complete profile of all transcripts present in an organism at any phase or time without leaving out any transcripts. Additionally, it lessens background noise and enhances experiment clarity. However, RNAseq analysis of NGS data is time-consuming because of variations in nucleotide composition between genomic areas and inconsistent read coverage across the genome. In RNAseq, a lengthy transcript is anticipated to contain more reads than a short transcript. Some software programs that display RNAseq data via transformed values, such as Reads per kilo base per million reads (RPKM) or the related Fragments per kilo base of transcript per million mapped fragments (FPKM), are used to normalize the counts in proportion to transcript length [199]. For example, the Cufflinks/Cuffdiff software provides an integrated analysis pathway from aligned reads to differential expression data, with inference based on FPKM values. RNAseq advancements will transform plant transcriptomics research, paving the path for generating agricultural cultivars that can withstand biotic and abiotic stresses.

The upcoming trend to study the transcriptional information of individual cells by single-cell RNA sequencing (scRNA-seq) is going to revolutionize applied plant research. This approach is being utilized in various model crops, including *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Nicotiana attenuate* and *Solanum lycopersicum* [200–204]. The increased application of these technologies in modal plant species will pay the way for its use in crop species to benefit agricultural systems.

Upcoming proteomics techniques

Proteomic analysis is crucial for crop development since it complements transcriptomics and metabolomics in elucidating plant cellular mechanisms. Plant biology has been unravelled thanks to recent advances in proteomics techniques. However, there are a number of limitations to these strategies that must be overcome to develop smart crops with excellent grain quality that can withstand various environmental stresses. Peptidomics, phosphoproteomics and redox proteomics are examples of new emerging technologies that will provide in-depth insight into molecular interactions and protein function [205]. Using modern proteomic technologies, we can generate more stress-tolerant or stress-adaptive cultivars. Activity based protein profiling (ABPP) is a very powerful and elegant and simple chemical-proteomics technique for studying the activities of 'active proteome' and allows multiplexing of different probes specific to proteasome, Ser hydrolases, Cys proteases, glutathione transferases, aldehyde hydrogenases, metalloproteases, ATP-binding proteins, α-glycosidases and β-glycosidases in plants [27, 206]. There is a huge scope for ABPP as it overcomes the challenges of studying the 'active proteome', which is very dynamic owing to phenomena like post-translational modifications, apoplast manipulation during infection, and changes in activities of enzymes due to physiological conditions [206]. It can overcome the limitations of conventional genomics and proteomics approaches to a large extent.

Artificial intelligence and phenomics

Big data, storage problems and the extraction of useful information have been caused by the continued usage of aerial and ground-based high throughput platforms with a range of image sensors at various locations during different periods of the plant's growth. Deep learning and machine learning technologies are utilized to overcome this problem by producing trustworthy results from massive data sets. Machine learning (ML), artificial intelligence (AI) and the Internet of Things (IoT) are interdisciplinary approaches to data analysis that use probability, statistics, classification, regression, decision theory, data visualisation and neural networks to link data extracted with the phenotype obtained. ML gives plant breeders, pathologists and agronomists a considerable edge by allowing them to extract multiple parameters for examining each trait simultaneously compared to previous methods that only examined one feature at a time. Another major machine learning advance links variables collected from HTP data to plant stressors, biomass accumulation, grain yield and soil properties. Machine Learning models automatically learn patterns from a large data set using non-linear activation functions [35]. Multilayer perceptron, generative adversarial networks, convolutional neural networks and recurrent neural networks are some key DL models utilised in phenomics [207]. These possible data analysis tools will help to expand the scope of HTP's possibilities in plant breeding.

Acknowledgements The authors are highly grateful to the SKUAST-K authorities especially Hon'ble VC Prof. Nazir A. Ganai, for promoting plant biotechnology and encouraging critical thinking among students.

Author contributions AMH conceptualized, analysed and edited the manuscript. SAH and TB wrote the paper with critical inputs from THR and AMH. All authors contributed to the article and approved the submitted version.

Funding The authors declare that no funds, grants or other support were received during the preparation of this manuscript.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Competing interests The authors have no conflict of interest and nothing to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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REVIEW ARTICLES

The Himalayan rhubarb (*Rheum australe* D. Don.): an endangered medicinal herb with immense ethnobotanical 'use-value'

Arshy Hussain[1,2] · **Mehak Aslam**[1,2] · **Tariq H. Masoodi**[2] · **M. Ashraf Bhat**[1] · **Prasada R. Kongala**[3] · **Amjad M. Husaini**[1]

Received: 20 September 2023 / Revised: 3 March 2024 / Accepted: 23 March 2024

Abstract

Rheum australe is a high-altitude medicinal plant found in the Himalayan region. It has huge therapeutic and economic potential owing to the occurrence of various phytochemical compounds like anthraquinones, stilbenes, tannins and flavonoids in the rhizome of the plant. The species possesses a wide variety of pharmacological activities like anti-cancer, antioxidant, anti-inflammatory, anti-microbial, anti-fungal, anti-diabetic, anti-ulcer, hepatoprotective, immune-enhancing and nephroprotective activities. It is used to treat various ailments like constipation, indigestion, diarrhoea, muscular pain, skin problems, and cough in different conventional systems of medicine. Due to its high therapeutic potential and use in the pharma industry, the species is overexploited in its natural habitats, rendering it endangered. Therefore, immediate efforts are required for its multiplication and conservation using biotechnological approaches like plant tissue culture.

Keywords *Rheum emodi* Wall · Pharmacological activities · Phytochemistry · Medicinal value

Introduction

Rheum australe D. Don. (also known as *Rheum emodi* Wall. ex Meissn.) is a perennial robust herb with significant therapeutic potential, commonly referred as Himalayan Rhubarb and Red-veined Pie Plant. It belongs to Kingdom: Plantae; Division: Magnoliophyta; Class: Magnoliopsida; Order: Caryophyllales; Family: Polygonaceae; Genus: Rheum L.). The species is naturally found in India, Myanmar, Nepal, Pakistan, Bhutan and China (Fig. 1a). In India, the species occurs in temperate Himalayas within altitudinal range of 2800–4000 m. In Kashmir, it is found in high-altitude areas like Gulmarg, Sonamarg, Tosamaidan, Dhara, and Pahalgam (Fig. 1b). Known by the name of "Pambchallan", it is an important source of food and medicine in forest fringe and high-altitude places of Kashmir. The leaves are cooked as a vegetable, locally known as ''Pambhaak''. The plant is used as an antiseptic and for treating frost bites in harsh winters. In addition, pies of the plant that serve as antipyretic and anthelmintic are made and are used to cure constipation, jaundice, and liver disorders.

R. australe is used in different traditional systems of medicine to cure a range of ailments and is also cited in various ancient manuscripts. The medicinal importance of *R. australe* has been documented from different parts of the world for different kinds of ailments like gastritis, stomach problems, menstrual problems and liver diseases (Alam et al. 2005). Various scientific studies have been carried out on the medicinal aspect of the plant. The rhizome of the plant is reported to contain a wide variety of chemical compounds like anthraquinones and stilbenes which possess various pharmacological activities like antiseptic, anti-inflammatory, anti-oxidant and anti-fungal activity. Due to various phytochemicals and their therapeutic potential, the plant has been heavily exploited for crude drugs in recent years, rendering this species endangered (Parveen and Wani 2013) (Fig. 2). Various other factors, such as poor seed germination and seedling survival in nature, have also rapidly declined their wild population. Therefore, the species has been recognized as the main strategic species for conservation, multiplication

✉ Amjad M. Husaini
amjadhusaini@skuastkashmir.ac.in

[1] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, SKUAST-K, Shalimar, Srinagar, Jammu and Kashmir, India

[2] Division of Forest Products and Utilization, Faculty of Forestry, SKUAST-K, Benhama, Ganderbal, Jammu and Kashmir, India

[3] Newe Yaar Research Centre, PO 1021, 30095 Ramat Yishai, Israel

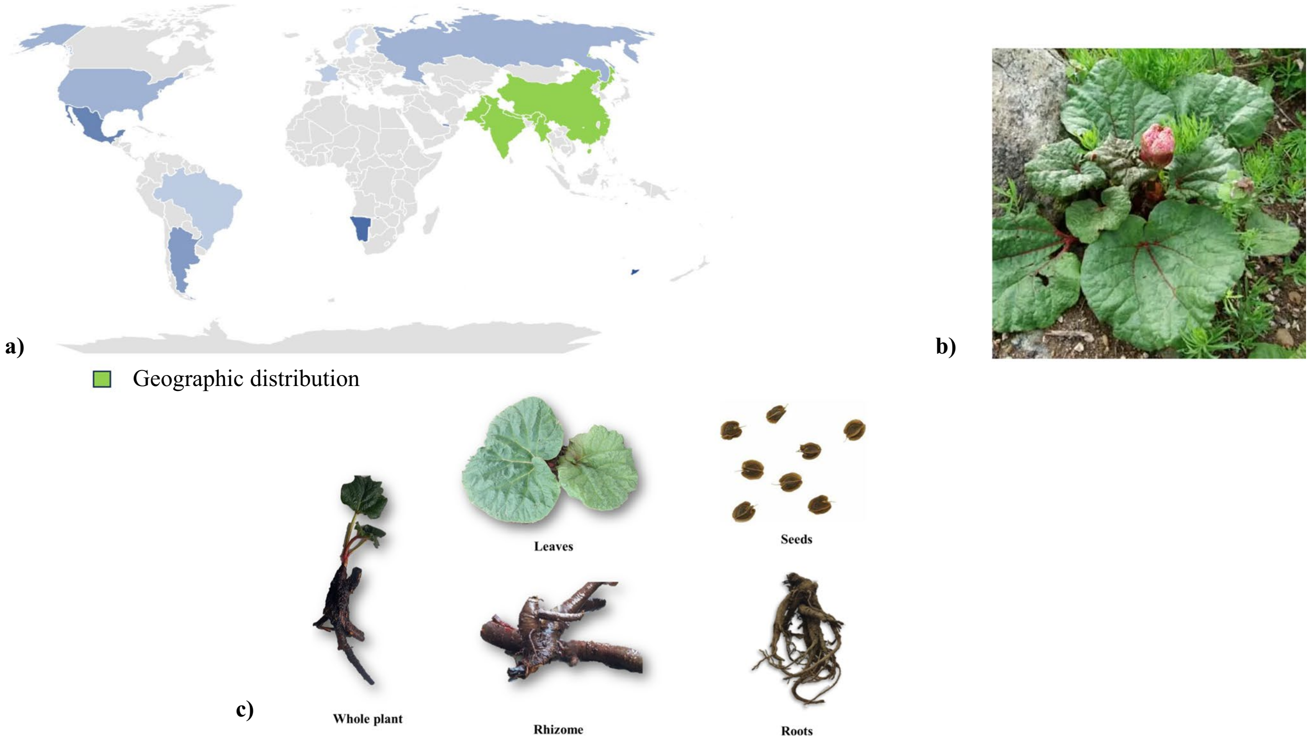

Fig. 1 *Rheum australe*: **a** Geographic distribution, **b** Natural habitat, **c** Morphology

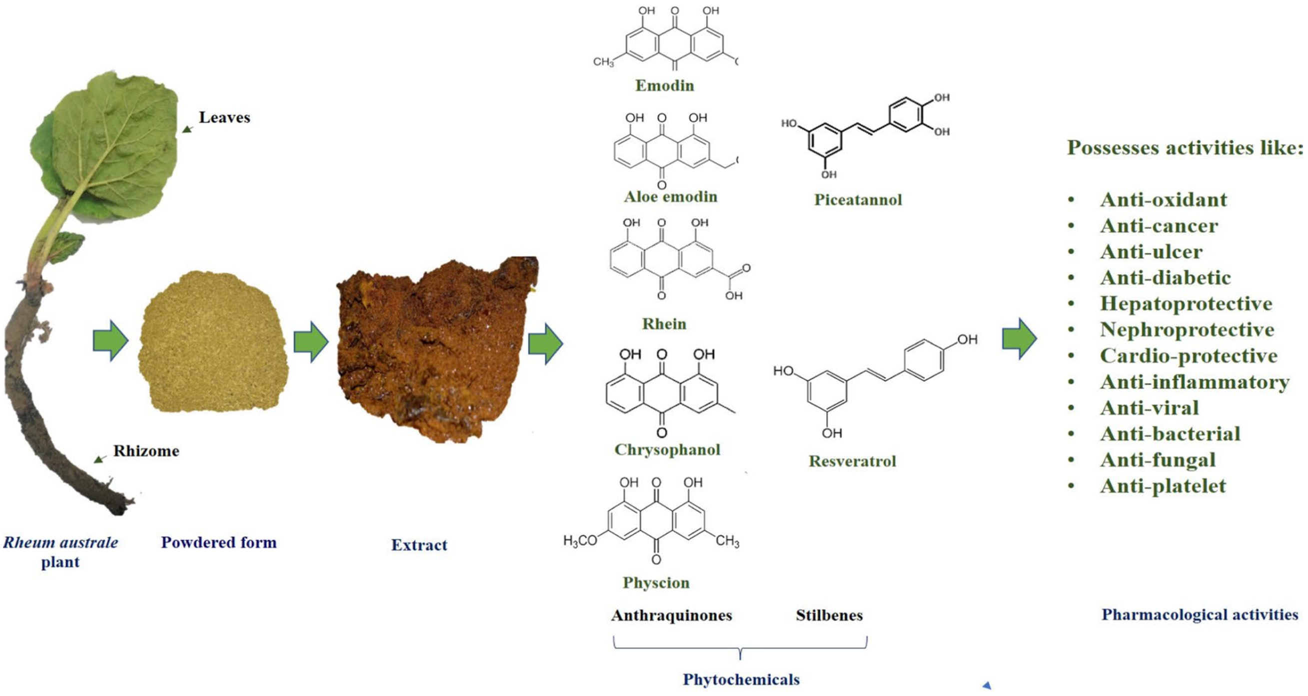

Fig. 2 Therapeutic effects of *Rheum australe* bioactive compounds

and domestication to reduce pressure on natural habitats (KabirDar et al. 2014).

Botanical description and cultivation

Rheum australe belonging to the family Polygonaceae, is a rhizomatous perennial herb with thick leafy stalks (Fig. 1c). The stem is smooth or pubescent, streaked green and brown with purple to red shade. The leaves are streaked, wrinkled with a rough surface, greenish-red in colour and heart-shaped with a roundish base. The upper leaves are smaller, while basal leaves are quite large, even up to 60 cm across with thick blades. The flowers are small, dark reddish-purple or yellow, formed in a long panicle inflorescence in densely-branched clusters. Flowering occurs in the month of June-July. The inflorescence can reach one foot in length and enlarges greatly when it grows into a fruit. Fruit is small (about 13 mm long), purple-coloured and ovoid-oblong, ovoid-ellipsoid or broadly ellipsoid in shape. The fruit has cordate base and notch at apex with narrow wings. The seeds are formed in late August till mid of September and harvested when they turn dark brown in colour (Rajkumar et al. 2011). Seeds are winged. Rhizomes and roots are collected from October to November and are the main parts containing drug. Rhizomes are thick and strong an d have a dark brown colour. The colour of a freshly cut surface ranges from dull orange to yellowish brown. The plant grows 1.5–3.0 m tall and is found on grassy slopes, close to water, and on rocks in debris and fissures.

R. australe can be cultivated either through seeds or by vegetative means using rhizome of the plant. The germination and seedling survival rates are reported to be poor. However, mature seeds show better germination when soaked in water for 10–12 h prior to sowing. The plant can be successfully propagated from rhizome segments (Singh et al. 2017). The plant exhibits strong growth and adaptability in areas of the Himalayas characterized by rocky landscapes and some shade, particularly in alpine and sub-alpine regions. The soil should be well-drained, porous, and rich in humus.

Traditional uses

The word rhubarb has been derived from Latin word “rha” meaning “river” and “barb” meaning “barbarian land”. In ancient times, rhubarb roots were imported by Romans from barbarian lands across Volga or Rha River thus the plant became Rhabarbarum. According to Lindley's Treasury of Botany, the name has been derived from Greek word “rheo” which means 'to flow', in reference to the purgative properties of the root. The medicinal use of *Rheum emodi* can be traced back thousands of years, as it is documented in “The Shen Nong Ben Cao Jing”, the earliest book on materia medica in the world. The medicinal properties of Rhubarb have been well known to the Chinese from a period long before Christian era around 2700BC. The roots of *Rheum emodi* were used in Europe and Middle East even during the Middle Ages and are used in Chinese medicine even today. During first century, rhubarb was being imported in Greece and Rome. By the tenth century, it had become a major export of Asia. In 1542, the price of rhubarb root was ten times the price of cinnamon and four times that of saffron in France. In Europe, it was costlier than opium. Dioscorides and Galen reported the medicinal properties of rhubarb in their manuscripts, and Avicenna has cited the use of rhubarb in almost all systems of the body (Malik et al. 2016).

Rheum australe has been extensively used as an herbal medicine for years without any antagonistic effects in numerous traditional systems of medicine. (Nazir et al. 2013) (Table 1). (Singh and Chaturvedi 2018) stated that Rhubarb is used as an ingredient in various Ayurvedic medicines like Chinimco tablet, Arjin tablet, Bleminor cream, Kankayan Vati and Panchamla taila. Traditionally, the plant is reported to be used as a laxative, tonic, and diuretic. The powdered root is used to clean teeth and is spread over wounds for

Table 1 Traditional uses of *Rheum australe*

Diseases	Mode of application	Reference
Inflammation, redness and swelling	Its oil is applied to the affected part	(Malik et al. 2016); (Sina 2010)
Indigestion	Taken with lukewarm water or *Aloe vera*	(Malik et al. 2016)
Diarrhoea	It can be taken with gum of acacia and dried roses	(Daniyal et al. 2019)
Paralysis, migraine and headache	Taken with Aloe vera	(Singh et al. 2017)
Skin marks and freckles	Paste of *Rheum australe* root mixed with vinegar is applied to the affected part	(Malik et al. 2016)
Bruises	Taken with grapes water	(Sina 2010)
Haemoptysis	The crushed root is chewed for some time	(Sina 2010)
Asthma	Crushed rhubarb is taken with resins of Quince	(Sina 2010)
Cough and rhinitis	The root powder is used with honey	(Singh et al. 2017)

rapid healing. It is widely used to treat fever, skin sores, cough, diarrhoea and indigestion (Kaur et al. 2015). Besides its medicinal uses, Rhubarb has also been used for its tart flavour in jams, jelly and sauce (Malik et al. 2016) and as an eco-friendly dye for the colouration of textile and wooden material (Das et al. 2008). It gives a golden colour to hair so it can also be used as bleaching agent. Leaf-stalk, leaves and flowers of Rhubarb can also be consumed as vegetables (Wani et al. 2009).

Pharmacological activities and phytochemistry

Rheum australe has a diverse pharmacological spectrum. Studies have reported many bioactive effects of Rhubarb, such as anti-bacterial, anti-inflammatory, anti-fungal, anti-cancer, anti-oxidant, anti-coagulant and anti-platelet activities (Fig. 3; Table 2). The reported activities are linked to various phytochemicals present in the rhizome of the plant. Rhubarb contains a wide array of phytochemicals like anthraquinones, stilbenes, flavonoids, tannins, saponins and volatile oils (Nazir et al. 2013) (Table 3). Among all, the most common phytochemicals present are anthraquinones (emodin, rhein, aloe-emodin, chrysophanol, physcion, and their glycosides) and stilbenes (piceatannol, resveratrol and their glycosides) (Kaur et al. 2015). Research studies have revealed the presence of anthrone C-glucosides and different oxanthrone derivatives viz. revandchinone-1, revandchinone-2, revandchinone-3 and revandchinone-4 in the rhizome of *Rheum emodi* (Krenn et al. 2003; Babu et al. 2003). Moreover, compounds like naphthoquinones, rutin, rheinal, and epicatechin have also been isolated (Singh et al. 2005). Additionally, two rare auronols, carpusin and maesopsin, were isolated from *Rheum emodi* (Krenn et al. 2003).

- Anti-diabetic activity

  The anti-diabetic properties of rhubarb might be associated with the occurrence of high quantities of flavonoids and anthraquinones (Xue et al. 2010). Chrysophanol and chrysophanol-8-O-b-D-glucopyranoside have been shown to augment the effect of insulin on glucose absorption by increasing the expression of Glut4 and insulin receptor phosphorylation (Lee and Sohn 2008). Du et al. (2012) reported that rhein boosts glucose tolerance in db/db mice by restoring their ability to produce insulin in the early stages. It was reported that rhein

Fig. 3 *Rheum australe* pharmacological activities

Table 2 Pharmacological activities of different extracts of *Rheum australe*

Extract/ solvent	Pharmacological activity	Reference
Ethanolic	Anti-diabetic	(Ravindran et al. 2010)
	Antidyslipidemic	(Mishra et al. 2014)
	Anti-oxidant	(Chai et al. 2012)
	Anti-ulcer	(Kaur et al. 2012)
Methanolic	Anti-cancer	(Pandith et al. 2014); (Kuo et al. 2002); (Rajkumar et al. 2011)
	Anti-inflammatory	(Chauhan et al. 2008)
	Anti-fungal	(Agarwal et al. 2000)
	Hepatoprotective	(Akhtar, MS et al., 2009)
	Anti-diabetic	(Arvindekar et al. 2015)
	Anti-oxidant	(Rajkumar et al. 2011)
	Nephroprotective	(Alam et al. 2005)
Ethyl acetate	Anti-cancer	(Pandith et al. 2014); (Zhang et al. 2015)
	Anti-platelet aggregation and anti-coagulant	(Seo et al. 2012)
	Anti-oxidant	(Chai et al. 2012); (Park and Lee 2021)
n-butanol	Nephroprotective	(Zhang et al. 2015)
	Anti-oxidant	(Chai et al. 2012)
	Anti-platelet aggregation and anti-coagulant	(Seo et al. 2012)
Petroleum ether	Anti-inflammatory	(Ahmad et al. 1998); (Zhang et al. 2015); (Chauhan et al. 2008)
	Anti-oxidant	(Chai et al. 2012)
Chloroform	Anti-cancer	(Kumar et al. 2012)
Hexane	Anti-platelet aggregation and anti-coagulant	(Seo et al. 2012)

treatment significantly boosted glucose-dependent and independent secretion of insulin in db/db mice by protecting β-cell mass and preventing β-cell death. These properties of rhein could make it a contemporary therapeutic agent for the prevention or treatment of diabetes in near future. In addition, rhein can guard the kidneys from developing diabetic nephropathy due to its antioxidant and anti-inflammatory properties (Zeng et al. 2014). Flavonoids produce antihyperglycemic effects by reducing the amount of glucose absorbed from the small intestine, improving tissue glucose uptake, decreasing gluconeogenesis, promoting release of insulin from β cells, and upholding the mass of β cells (Ghorbani 2017). Arvindekar et al. (2015) reported that Himalayan Rhubarb contains alpha-glucosidase inhibitors which help in decreasing the intestinal absorption of glucose. The rhizomes of Rhubarb also contain stilbene compounds which have anti-diabetic properties. Ravindran et al. (2010) conducted a study to assess the anti-diabetic effects of *Rheum emodi* rhizome extract and to investigate the activities of various glycolytic and gluconeogenic enzymes in the liver and kidney of normal and alloxan-induced diabetic rats. They reported that administration of *Rheum emodi* rhizome extract improved peripheral glucose utilization correcting glycolytic disorders in the liver and kidneys. The hexokinase and phosphoglucose isomerase activities were significantly decreased, while aldolase activity was significantly increased in diabetic rats in comparison to control rats. The activities of gluconeogenesis enzymes, glucose-6-phosphatase and fructose-1,6-bisphosphatase (liver and kidney) were significantly increased in diabetic rats compared to control rats. Oral administration of *Rheum emodi* (250 mg/kg body weight) for 30 days significantly reversed these values to normal. Moreover, administration of *Rheum emodi* rhizome extract led to a significant decline in blood glucose level in comparison to diabetic control rats.

- Nephroprotective activity

Nephrotoxicity is often induced by a wide spectrum of substances like antibiotics, heavy metals, therapeutic drugs, and biological agents, which tend to accumulate in the kidneys and cause extensive structural and functional effects on the kidneys (Alam et al. 2005; Barnett and Cummings 2018). Nephrotoxicity is characterized by the reduction in glomerular filtration rate (GFR), which is clinically assessed by increased serum creatinine (SCr) and decreased creatinine clearance or decreased urine output (Isiiko et al. 2021).

Rhubarb has been proven to possess nephroprotective and anti-fibrotic activities in patients with chronic kidney diseases (Zhang et al. 2015). Previous researches indicate that emodin and quercetin might be the nephroprotective compounds in rhubarb (Waly et al. 2013; Shin et al. 2015). Emodin has been reported to be an antioxidant by

Table 3 Phytochemicals of *Rheum australe* and their pharmacological activities

S. No	Pharmacological activity	Compound	Gist of main findings	References
1	Anti-diabetic activity	Chrysophanol and chrysophanol-8-O-b-D-glucopyranoside	Augment the effect of insulin on uptake of glucose by increasing expression of Glut4 and insulin receptor phosphorylation	(Lee and Sohn 2008)
		Rhein	Enhances glucose tolerance by restoring early insulin production in db/db mice	(Du et al. 2012); (Zeng et al. 2014)
			Protects the kidneys from development of diabetic nephropathy	
		Flavonoids	Produce antihyperglycemic effects by reducing the amount of glucose absorbed from the small intestine, improving tissue glucose uptake, decreasing gluconeogenesis, promoting insulin release from β cells, and upholding the mass of β cells	(Arvindekar et al. 2015)
		Alpha-glucosidase inhibitors	Help to decrease the intestinal absorption of glucose	(Arvindekar et al. 2015)
2	Nephroprotective activity	Emodin	Reduces the toxicity of nephrotoxic drugs such as cisplatin for human kidney cells (HEK 293)	(Waly et al. 2013)
		Quercetin	Protects the kidneys from mercury toxicity	(Shin et al. 2015)
3	Anti-oxidant activity	Piceatannol-4 -O-β-D-glucopyranoside (PICG) and piceatannol (PICE),	Both PICG and PICE demonstrated positive antioxidant action with PICE exhibiting strong antioxidant activity	(Chai et al. 2012)
		Phenolic compounds	Different phenolic compounds present were found to be strongly correlated with free radicals scavenging efficacies, percentages of LPI and Fe3 + reductions	(Rajkumar et al. 2011)
		Myricitrin, myricetin 3-galloyl rhamnoside, and myricetin,	These compounds were recognized as antioxidants and have not been stated in previous studies about *R. emodi*	(Park and Lee 2021)

Table 3 (continued)

S. No	Pharmacological activity	Compound	Gist of main findings	References
4	Anti-cancer activity	Emodin	It exerts an inhibitory effect on the invasiveness (metastasis) of different human breast cancer cell lines like MCF-7 cells Emodin induces gene silencing in bladder cancer cells and also reverses the cancer epigenetics towards normal epigenetic conditions Emodin could significantly suppress the colony development and proliferation of various cancer cell lines viz. human lung adenocarcinoma cells (A549 cells), hepatoma cells, human cervical cancer cell line HeLa, human colon cancer HCT116 cells, and LOVO colorectal cancer cells by inducing apoptosis and interfering in some oncogenic signalling pathways Enhances the efficiency of cisplatin against human gastric cancer cell (SNU-5) Emodin suppressed the self-renewal activity of glioma stem cells invitro It sensitizes the tumor cells to chemotherapeutic agents	(Li et al. 2013); (Cha et al. 2015); (Li et al. 2014); (Subramaniam et al. 2013); (Yaoxian et al. 2013); (Xie et al. 2014); (Maa 2014); (Huang et al. 2015); (Kim et al. 2015); (Dong et al. 2016)
		Aloe emodin	Aloe-emodin inhibits cell growth in several tumor cells, including human lung carcinoma (CH27), liver carcinoma (Hep G2 and Hep 3B), HPV-induced cervical cancer cells, gastric and leukaemia cell lines Both emodin and aloe-emodin suppress ER α transcriptional activation thereby inhibiting breast cancer cell proliferation	(Lee et al. 2001); (Kuo et al. 2002); (Chihara et al. 2015); (Chen et al. 2004); (Huang et al. 2013)
		Rhein	Rhein inhibits proliferation of various cancer cell lines including human breast, lung, colon, CNS, and glioma cancer cells by modulating different signalling cascades in cancer cells It prevents glucose absorption in tumour cells and induce cell necrosis	(Cichewicz et al. 2004); (Henamayee et al. 2020); (Huang et al. 2007)

Table 3 (continued)

S. No	Pharmacological activity	Compound	Gist of main findings	References
5	Hepatoprotective activity	Rhein	Protects cholangiocytes and hepatocytes from α-naphthyl isothiocyanate induced damage in rats	(Zhao et al. 2009)
			Rhein may ameliorate methotrexate-induced liver injury	(Bu et al. 2018)
		Emodin	Treatment with emodin shows dose dependant decrease in acetaminophen induced hepatotoxicity in rats	(Bhadauria 2010)
		Aloe-emodin and physcion	Can also protect cholangiocytes and hepatocytes from α-naphthyl isothiocyanate induced damage in rats	(Zhao et al. 2009)
6	Neuroprotective activity	Emodin	Emodin shows neuroprotective effects against β-amyloid-induced neurotoxicity in rat cortical neurons	(Liu, Mi-Hua et al., 2016); (Li et al. 2018); (Lu et al. 2005)
			It exerts neuroprotective effect by regulating the expression of connexin 43 and aquaporin 4 in cerebral ischemia/reperfusion rats	
			Emodin enhanced the neurological symptom evaluation score, brain water ratio, and cerebral infarction area in model rat	
		Aloe emodin	Aloe- emodin has neuroprotective effects against Alzheimer's disease by inhibiting acetylcholinesterase activity and modulating oxidative stress	(Tao et al. 2014)
			It also decreases cytotoxicity induced by H_2O_2 in PC12 cells and scopolamine-induced cognitive deficits in mice	
7	Anti-viral activity	Emodin	It exhibits the ability of inhibiting infection and reproduction of more than 10 viruses in vitro and in vivo	(Shao et al. 2022); (Ho et al. 2007); (Schwarz et al. 2011); (Nawrot-Hadzik et al. 2021)
			Emodin can become a new anti-SARS CoV compound by blocking the interaction of S protein and host cell receptor	
			Emodin can hinder the 3a ion channel of coronavirus SARS-CoV and HCoV-OC43	
			Emodin is reported to inhibit over 50% of SARS-CoV-2 Mpro	

Table 3 (continued)

S. No	Pharmacological activity	Compound	Gist of main findings	References
8	Cardioprotective activity	Emodin	Emodin has shown cardioprotective activity after ischemia–reperfusion by differentially enhancing mitochondrial antioxidant components	(Du and Ko 2006); (Zhang et al. 2016);
			Emodin reduced cardiac damage in mice infected with Coxsackie virus B3 by reducing the expression of P38 mitogen activating protein kinase and Toll-like receptor 4	
		Resveratrol	Protected H9c2 cells against doxorubicin-induced damage via SIRT1 activation and reducing oxidative damage in Wistar rats	(Liu, M. H. et al., 2016); (Tatlidede et al. 2009)
9	Anti-ulcer activity	Chrysophanol and emodin	Exhibited strong anti-ulcer activity against different types of ulcers in rats caused by ethanol, aspirin, pyloric ligation, and cold restraint	(Mishra 2016)
10	Anti-inflammatory activity	Emodin	Emodin exerts anti-inflammatory effects by blocking MAPK and PI3K signalling pathways and inhibiting the expression of NF-κB and iNOS	(Zhu et al. 2011); (Zhang et al. 2005)
			Emodin shows the inflammatory response in the rat lung by decreasing the expression of tumor necrosis factor-α (TNF-α) and IL-6	
		Aloe emodin	Aloe emodin derivatives demonstrated strong anti-inflammatory activity in Lipopolysaccharide-stimulated RAW 264.7 cell lines by inhibiting the production of Interleukin 6 (IL-6)	(Dileep Kumar et al. 2022)
		Chrysophanol	It is the main component of petroleum ether extract of rhizomes of *Rheum emodi* responsible for anti-inflammatory activity	(Ahmad et al. 1998)
		Rhein	Rhein also exhibits anti-inflammatory effects by inhibiting the expression of iNOS	(Wang et al. 2002); (Ge et al. 2017)
			Rhein may exhibit anti-inflammatory effects by inhibiting NF-κB and NALP3 inflammasome pathways	
			In addition, treatment with rhein reduced the production of proinflammatory cytokines, including IL-6, IL-1β	

Table 3 (continued)

S. No	Pharmacological activity	Compound	Gist of main findings	References
11	Anti-platelet and coagulation activity	Chrysophanol-8-O-glucoside	Inhibits platelet aggregation induced by collagen and thrombin In coagulation assays, it prolongs activated partial thromboplastin time, but does not affect prothrombin time	(Seo et al. 2012)

scavenging free radicals and elevating enzymes like catalase, glutathione peroxidase, glutathione S-transferase, glutathione reductase and superoxide dismutase that reduce the toxicity of nephrotoxic drugs such as cisplatin for human kidney cells (HEK 293) (Waly et al. 2013). Quercetin has also been revealed to protect the kidneys from mercury-induced toxicity by removing free radicals and inhibiting apoptotic cells (Shin et al. 2015). Alam et al. (2005) studied the renal effects of water-soluble and insoluble parts of the alcohol extract of *Rheum emodi* on mercuric chloride, cadmium chloride, potassium dichromate and gentamicin-induced nephrotoxicity in rats and normal rats by monitoring the level of urea nitrogen and creatinine in serum. They reported the nephroprotective effect of water-soluble fraction on all the proximal tubule segments (S1, S2 and S3) probably due to antioxidant activity of tannins ocuuring in the fraction. Water insoluble fraction was also reported to improve the renal function of S2 segment of proximal tubule by preventing nephrotoxicity induced by metals viz cadmium chloride and mercuric chloride in rat models, but, this portion has been found to augment gentamicin induced nephrotoxicity.

- Anti-oxidant activity

  Oxidative stress is one of the factors that contributes to the progression and emergence of several fatal diseases like cancer, diabetes, brain degeneration, and hepatotoxicity. Plant-based antioxidants could be useful in their prevention and treatment (Nazir et al. 2013). Several studies have demonstrated the anti-oxidant activity of *Rheum emodi*. The anti-oxidant properties of *Rheum emodi* are due to the incidence of various anthraquinones and stilbenes in the plant. Anthraquinone compounds emodin, physcion, aloe-emodin and chrysophanol isolated from Rheum emodi rhizome are reported to have anti-oxidant activity (Tripathi et al. 2014). In the past few years, plant stilbenoids have drawn significant consideration as a source of antioxidants. The anti-oxidant activity of stilbenoid piceatannol-4 -O-β-D-glucopyranoside (PICG) and piceatannol (PICE) isolated from the rhizome of *Rheum emodi* and was studied by evaluating DPPH and superoxide anion radical scavenging, ferric reducing power and inhibition of lipid peroxidation in vitro. Both PICG and PICE demonstrated positive antioxidant action in each of the four assays. It was further reported that PICE has strong antioxidant activity as it contains 3 -hydroxyl group which may boost the antioxidant effects of stilbenoids (Chai et al. 2012). The extracts of *Rheum emodi* were evidenced to comprise a high level of different phenolic compounds, which are closely linked with free radicals (DPPH and OH) scavenging efficacies, percentages of LPI and Fe3 + reductions (Rajkumar et al. 2011). A recent study advocates that total polyphenol content is significantly correlated with antioxidant activity screening analyses. Moreover, the ethyl acetate portion of *R. emodi* holds the highest antioxidant potential. The compounds associated with the antioxidant effects of *R. emodi* were recognized as myricitrin, myricetin 3-galloyl rhamnoside, and myricetin, and are not mentioned in previous studies about *R. emodi* (Park and Lee 2021).

- Anti-cancer activity

The main anthraquinone compounds in *Rheum*, emodin, aloe-emodin and rhein are reported to possess anti-cancer activity, inhibiting the growth and spread of cancer cells. Emodin exhibits potent anti-cancer activities by inhibiting the spread of various cancer cell lines such as lung, breast, cervical, colon, and prostate cancer cells (Li et al. 2014, 2013; Yaoxian et al. 2013; Xie et al. 2014).

Emodin induces cytotoxic effects in human breast carcinoma by regulating the expression of genes related to apoptosis in a concentration-dependant manner. Various studies have reported that emodin is also involved in suppressing the invasiveness (metastasis) of different human breast cancer cell lines (Jelassi et al. 2013; Guo et al. 2013; Jia et al. 2014; Sun et al. 2015). It exerts an inhibitory effect on the colony formation of human breast carcinoma MCF-7 cells (Li et al. 2013). Cha et al. (2015) demonstrated the anti-cancerous action of emodin on bladder cancer cells by using microarray analysis and chIP assays. Microarray analysis established that oncogenic genes responsible for abnormal proliferation and survival were significantly suppressed by emodin. The chIP assays also presented that emodin-induced gene silencing in bladder cancer cells by suppressing the activity of pH3Ser10 and by increasing H3K27me3, suggesting that emodin converses the cancer epigenetics towards normal epigenetic conditions. Li et al. (2014) studied the cytotoxic mechanism of emodin on A549 human lung adenocarcinoma cells and suggested that emodin could significantly obstruct the growth and colony development of A549 cells depending upon concentration by modifying the external apoptotic pathways and inducing cell cycle arrest. Emodin has been reported to hinder proliferation and bring morphological changes in human cervical cancer cell line HeLa by inducing apoptosis through endogenous mitochondrial and exogenous death receptor pathways (Yaoxian et al. 2013).

Emodin can significantly potentiate TRAIL-induced apoptosis by inducing death receptors and downregulating cell survival proteins in hepatoma cells (Subramaniam et al. 2013). Emodin also enhances the efficiency of cisplatin against human gastric cancer cell (SNU-5) by inducing apoptosis and cell cycle arrest (Huang et al. 2015).

Emodin suppressed the self-renewal activity of glioma stem cells in vitro. It inhibited the upkeep of stemness by suppressing the expression of proteins involved in stemness signalling. Emodin further amplifies this effect by hindering the interaction between Hsp90 and EGFR/EGFRvIII (epidermal growth factor receptor/ epidermal growth factor receptor variant III), resulting in the degradation of EGFR/EGFRvIII (Kim et al. 2015).

Emodin induces apoptosis in human colon cancer HCT116 cells and LOVO colorectal cancer cell growth by regulating the mitochondrial apoptotic pathway (Xie et al. 2014; Maa 2014). Moreover, emodin also suppresses Wnt signalling pathway in human colorectal cancer cells (SW480 and SW620) by downregulating the transcriptional activity of T cell factor (Pooja and Karunagaran 2014).

The synergetic outcome of emodin and chemotherapy has been evaluated as it sensitizes the tumor cells to chemotherapeutic agents (Dong et al. 2016). Studies have revealed that normal cells are more resistant to emodin-induced toxicity than cancer cells. This specificity of emodin towards cancerous cells may be due to its interference with some oncogenic signalling pathways (He et al. 2019).

As demonstrated in multiple studies, aloe- emodin affects various vital pathways depending on cell type and exhibits numerous anti-proliferative and anti-carcinogenic properties in human cancer cell lines. The most prominent effects include inhibition of cell proliferation, migration, invasion and modulation of immune signalling. Aloe-emodin inhibits cell growth in several tumor cells like human lung carcinoma (Lee et al. 2001), liver carcinoma (Kuo et al. 2002), gastric (Chihara et al. 2015) and leukaemia cell lines (Chen et al. 2004). Aloe-emodin reportedly induced apoptosis in two human liver cancer cell lines, Hep G2 and Hep 3B and inhibited cell proliferation by different anti-proliferative mechanisms (Kuo et al. 2002). Aloe-emodin induces cell death in human lung squamous cell carcinoma (CH27) by the activation of caspases (caspase-3, caspase-8, and caspase-9) through Bax and Fas death pathway (Lee et al. 2001). The efficiency of aloe- emodin in preventing the proliferation of MCF-7 cells in a dose and time-dependent manner using WST-1 assay was demonstrated by (Amin et al. 2013). Recently, aloe-emodin was also found to induce apoptosis in HPV-induced cervical cancer cells (Gao et al. 2019). Aloe-emodin also exhibited high specificity for neuroectodermal tumour cells (Pecere et al. 2003). Both emodin and aloe-emodin are capable of suppressing ER α transcriptional activation by downregulating ER α protein levels, thereby inhibiting the proliferation of breast cancer cells (Huang et al. 2013). In addition, aloe- emodin, emodin and chrysophanol exhibited higher cytotoxicity against salivary gland tumor and oral squamous cell carcinoma than normal human gingival fibroblasts (HGF) (Shi et al. 2001).

Rhein has also been reported to inhibit the proliferation of various cancer cell lines including human breast, lung, colon, CNS, and glioma cancer cells by modulating different signalling cascades in cancer cells (Cichewicz et al. 2004; Henamayee et al. 2020). Rhein could efficiently prevent glucose absorption in tumour cells and induce cell necrosis (Huang et al. 2007). Rajkumar et al. (2011) evaluated the anti-cancer activity of two extracts (methanolic and aqueous) of *Rheum emodi* rhizome and reported their apoptosis induction potential in human breast carcinoma (MDA-MB-435S), prostate cancer (PC 3) and liver carcinoma (Hep3B) cell lines. The studies suggest that chloroform extracts of *Rheum emodi* could help in designing effective anti-cancer drugs, especially for advanced stages of breast cancer (Kumar et al. 2012).

- Hepatoprotective activity

Excessive medications, herbal supplements, chemicals, solvents, and alcohol often cause hepatotoxicity. The magnitude of hepatotoxicity is generally estimated by the level of glutamate pyruvate transaminase (ALT), glutamate oxaloacetate transaminase (AST), alkaline phosphatase (ALP), bilirubin, albumin, and whole liver homogenate (Zargar et al. 2011).

The hepatoprotective activity of plants of Rheum genus can be attributed to the presence of different phytochemical compounds like emodin, rhein, and resveratrol. The majority of the studies on the hepatoprotective effect of Rhubarb are conducted using chemical-induced liver damage in rodents. Various studies have reported the hepatoprotection of Rhubarb against CCl_4-induced toxicity in rats (Meesala et al. 2021; Hassan 2008; Ibrahim et al. 2008). Similarly, treatment with a methanolic extract of rhubarb has been shown to reduce serum albumin, bilirubin, AST and ALT levels in rats exposed to paracetamol-induced hepatotoxicity (Akhtar, Mymoona et al., 2009). Studies have also shown that phytochemicals of rhubarb exhibit hepatoprotective effect against naphthyl isothiocyanate (ANIT) and concanavalin A-induced liver injury in rats (Mase et al. 2010; Zhao et al. 2009). The hepatoprotective effects of free anthraquinones in rhubarb were investigated and results suggest that rhein, aloe-emodin and physcion can protect cholangiocytes and hepatocytes from α-naphthyl isothiocyanate- induced damage in rats. Rhein was found to be the most effective compound, whereas emodin and chrysophanol resulted in partial protection (Zhao et al. 2009). Treatment with emodin shows dose dependant decrease in hepatotoxicity by protecting various blood and tissue biochemical changes induced by acetaminophen in rats (Bhadauria 2010). Rhein may ameliorate methotrexate-induced liver injury by acting on the Nrf2-HO-1 pathway. Rhein significantly reduces alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and number of apoptotic cells in MTX-treated normal human hepatocytes (L02 cells) (Bu et al. 2018).

- Neuroprotective activity

Neurodegenerative diseases occur when neurons in the brain and spinal cord begin to deteriorate. Elevated levels of glutamate decrease antioxidant enzyme levels and increase ROS production, leading to neurodegenerative diseases (Michaels and Rothman 1990). Various anthraquinone derivatives of rhubarb, alone or in combination, exhibit neuroprotective effects through anti-inflammation, anti-oxidative stress, and maintenance of blood–brain barrier integrity in different diseases of central nervous system like cerebral ischemic stroke, traumatic brain injury, intracerebral haemorrhage, Alzheimer's disease, brain tumour and depression (Li et al. 2019). *Rheum emodi* exhibits neuroprotection against glutamate toxicity in IMR32 cells via upregulating Nrf2/HO-1 expression (Hamid et al. 2018). Emodin is reported to provide neuroprotection in rat cortical neurons against β-amyloid-induced neurotoxicity (Liu, Mi-Hua et al., 2016). Aloe- emodin could have neuroprotective effects against Alzheimer's disease by modulating oxidative stress and inhibiting the activity of acetylcholinesterase. It also decreases cytotoxicity induced by H_2O_2 in PC12 cells and scopolamine-induced cognitive deficits in mice (Tao et al. 2014). Emodin exerts neuroprotective effect in cerebral ischemia/reperfusion rats by regulating the expression of connexin 43 and aquaporin 4 (Li et al. 2018). In another in vivo study, emodin enhanced the neurological symptom evaluation score, brain water ratio, and cerebral infarction area in model rat by inhibiting the cascade inflammatory reaction (Lu et al. 2005).

- Anti-viral activity

Rhubarb is used as an anti-viral agent against *Varicella zoster* virus and *Herpes simplex virus rubella*. The minimal inhibitive doses were 100-5000 mg/L (Agarwal et al. 2001). (Shao et al. 2022) reviewed the anti-viral effect of emodin and summarized that it exhibits the ability to prevent infection and reproduction of more than 10 viruses in vitro and in vivo, such as herpes simplex virus type 1 and type 2 (Xiong et al. 2011); Bei et al., 2021), human cytomegalovirus (Barnard et al. 1992), Epstein-Barr virus (Yiu et al. 2022; Wu et al. 2019), coxsackievirus B (Liu et al. 2015), hepatitis B virus

(Shuangsuo et al. 2006), influenza A virus (Bei et al. 2021), SARS-CoV (Ho et al. 2007), viral haemorrhagic septicaemia rhabdovirus (Alves et al. 2004), enterovirus 71 (Zhong et al. 2017), dengue virus serotype 2 (Batool et al. 2018) and Zika virus (Batista et al. 2019). Emodin may be regarded as a potential therapeutic agent for the treatment of SARS and can become a new anti-SARS CoV compound by blocking the interaction of S protein and host cell receptor angiotensin-converting enzyme 2 in a dose-dependent manner. It also inhibits the infection by S protein-pseudo typed retrovirus (Ho et al. 2007). Emodin can prevent the 3a ion channel of coronavirus SARS-CoV and HCoV-OC43. It can also prevent virus release from HCoV-OC43 (Schwarz et al. 2011). Moreover, halogenation can improve the antiviral activity of emodin against human coronavirus NL63 (HCoV-NL63) (Horvat et al. 2021). (Nawrot-Hadzik et al. 2021) reported that emodin inhibits over 50% of SARS-CoV-2 Mpro.

- Cardio-protective effects

  The presence of emodin and resveratrol in the genus *Rheum* bestow cardio-protective effect in this herb (Negi et al. 2015). Emodin is known to mitigate cardiac damage after ischemia–reperfusion by differentially enhancing mitochondrial antioxidant components (Du and Ko 2006). According to (Zhang et al. 2016), emodin reduced cardiac damage in mice infected with Coxsackie virus B3 by reducing the expression of Toll-like receptor 4 and P38 mitogen activating protein kinase. (Liu, M. H. et al., 2016) demonstrated that resveratrol protected H9c2 cells against doxorubicin-induced damage via SIRT1 activation. By restoring SIRT1 activity to reduce USP7-related catabolic/pro-apoptotic signalling, resveratrol reduces the cardiotoxicity caused by doxorubicin in ageing hearts (Sin et al. 2015). Additionally by reducing oxidative damage, it has been proven to protect Wistar rats against cardiotoxicity caused by doxorubicin (Tatlidede et al. 2009).

- Anti-ulcer Activity

  The anti-ulcer activity of *Rheum emodi* rhizome extract has been reported in several studies. Chrysophanol and emodin exhibited strong anti-ulcer activity against different types of ulcers in rats caused by ethanol, aspirin, pyloric ligation, and cold restraint (Mishra 2016).

  Kaur et al. (2012) stated that ethanol extract of *Rheum emodi* rhizome at a dose of 100 mg/kg body weight decreased the ulcer index, volume and total acidity, and increased the pH of gastric fluid in pylorus-ligated rats. Additionally, it has gastroprotective and antioxidant effects because it lowers oxidative stress which improves the integrity of the gastric mucosa and increases the production of nitric oxide and mucus in experimentally generated gastric ulcers.

- Anti-inflammatory activity

  Studies have demonstrated that the phytochemicals found in *Rheum*, viz. emodin, aloe emodin, chrysophanol and rhein have anti-inflammatory properties. Emodin exerts anti-inflammatory effects by hindering MAPK and PI3K signalling pathways and inhibiting the expression of NF-κB and iNOS (Zhu et al. 2011). Emodin shows anti-inflammatory response in the rat lung by decreasing the expression of tumor necrosis factor-α (TNF-α) and IL-6 (Zhang et al. 2005). Aloe-emodin derivatives demonstrated strong anti-inflammatory activity in lipopolysaccharide-stimulated RAW 264.7 cell lines by inhibiting the production of Interleukin 6 (IL-6) (Dileep Kumar et al. 2022). According to Ahmad et al. (1998), chrysophanol is the main component of petroleum ether extract of rhizomes of *Rheum emodi* responsible for anti-inflammatory activity. Rhein also exhibits anti-inflammatory effects by inhibiting the expression of iNOS (Wang et al. 2002). Rhein may exhibit anti-inflammatory effects by inhibiting NF-κB and NALP3 inflammasome pathways. In addition, treatment with rhein reduced the production of proinflammatory cytokines, including IL-6, IL-1β, and tumor necrosis factor-α, were significantly reduced in lipopolysaccharide (LPS)-induced RAW264.7 macrophages (Ge et al. 2017). The methanolic and petroleum ether extract of rhubarb may be as effective as the non-steroidal anti-inflammatory drug ibuprofen in protecting albino rats against carrageenan-induced inflammation (Chauhan et al. 2008).

- Anti-platelet aggregation and anti-coagulant activities

  The anthraquinone derivatives present in rhubarb are reported to possess anti-platelet aggregation and anti-coagulant activity. Chrysophanol-8-O-glucoside inhibits collagen and thrombin-induced platelet aggregation, and the bleeding time of mice treated with it is significantly prolonged. In coagulation assays, it prolongs activated partial thromboplastin time, but does not change prothrombin time (Seo et al. 2012). Stilbenes present in Rhubarb have also been reported to possess an anti-platelet aggregation activity (Ko et al. 1999).

- Immune enhancing activity

  The *Rheum emodi* rhizome extract has been revealed to hold immuno-enhancing activity. This effect is reported to be due to a dose-dependent increase in nitric oxide and cytokine TNF-a, IL-12 and a reduction in IL-10 by RAW 264.7 macrophage cell lines in the presence of extract alone (Kounsar and Afzal 2010).

- Anti-bacterial activity

  Rhubarb is used to deal with gastrointestinal ailments since ancient times due to its anti-bacterial properties. Various extracts viz. ethanol, ether, petroleum, benzene, and chloroform extracts were tested

Table 4 Influence of different growth regulators and explants on micropropagation of *Rheum australe*

S. No	Explant used	Growth hormones	Response	Reference
1	Shoot tip (rhizome)	IBA (0.1–5 mg/l) BAP (0.5–5 mg/l) used alone and in combinations	Optimum no. of shoots with better growth in 4 weeks was observed in IBA (1.0 mg/l) + BAP (2.0 mg/l)	(Lal and Ahuja 1989)
	Leaf	IBA (1.0 mg/l) IAA (0.1–1.0 mg/l) BAP (0.5–5 mg/l)	Shoot buds developed into normal shoots within 6 weeks in IBA (1.0 mg/l) + BAP (2.0 mg/l) and IAA (0.25 mg/l) + BAP (2.0 mg/l)	
			Optimal root induction was observed at IBA (1.0 mg/l)	
2	Seeds	BAP (10 μM) 2, 4-D (5 μM) + TDZ (10 μM) Kn (10 μM) + NAA (10 μM) BAP (5 μM) + IBA (5 μM) and BAP (10 μM) Zeatin (10 μM) + IAA (2 μM)	Complete germination with whole plantlet formation was observed in BAP (5 μM) + IBA (5 μM) and BAP (10 μM)	(Tabin et al. 2014)
	Shoot tips, Nodal segments, Leaves, Roots, Rhizomes, Petioles	Zeatin (7 μM) + 2, 4-D (5 μM) BAP (5 μM) + 2, 4-D (10 μM) Kn (10 μM) + NAA (5 μM)	High callus was observed in Zeatin (7 μM) + 2, 4-D (5 μM)	
3	Shoot tips	BAP (2.5—15 μM) NAA (2.5—7.5 μM) 2,4-D (2.5, 5.0 μM) IAA (2.5, 5.0 μM) IBA (2.5–7.5 μM)	Longer shoots and optimum shoot multiplication was observed in IBA (2.5 μM) + BAP (2.5 μM) and IBA (5.0 μM) + BAP (7.5 μM) respectively	(Parveen and Kamili 2012)

to evaluate the anti-bacterial properties of *Rheum emodi* in male Wister rats. These extracts suppressed the growth of *H. pylori* in vitro and in vivo, and the contamination cleared within a week at very low concentrations. Also, the bacteria were not able to attain resistance against *Rheum emodi* extracts even after being exposed 10 times in a row (Ibrahim et al. 2006). Agarwal et al. (2001) reviewed the anti-bacterial properties of *Rheum* and stated that it is used against *Escherichia coli*, *Staphylococcus aureus* and *Brucella abortus*. Babu et al. (2003) also evaluated anti-bacterial properties of *Rheum emodi* against different gram- positive and gram-negative bacteria.

- Anti-fungal activity

  The presence of anthraquinone derivatives in *Rheum emodi* contributes to its ability to fight against fungal infections. Rhein, chrysophanol, aloe-emodin and physcion found in *Rheum emodi* rhizomes have been shown to fight against fungal infections caused by *Candida albicans, Cryptococcus neoformans, Trichophyton mentagrophytes, and Aspergillus fumigatus* (Agarwal et al. 2000). In another study, the anti-fungal activity of two different extracts (aqueous and methanol) of *Rheum emodi* against two fungal strains *Fusarium solani* MTCC3871 and *Aspergillus flavus* MTCC1037 were carried out and the extracts showed significantly high zones of inhibition against both the fungal strains (Malik et al. 2018). *Rheum emodi* rhizomes were also found effective against *Aspergillus niger* and *Rhizopus oryzae* (Babu et al. 2003).

- Miscellaneous activities

  Rheum emodi is an effective herb for relieving the symptoms of primary dysmenorrhoea. Rehman et al. (2015) reported that intake of *Rheum emodi* powder two days before the expected time of menstruation and continued for the first three days of menstruation two times a day, significantly decreased the severity of menstrual pain after the three-cycle intervention. Other associated symptoms, such as duration of pain and QOL (quality of life) were markedly enhanced after treatment. Rhubarb has also been reported to hold purgative properties (Li et al. 2008). Emodin, chrysophanol, emodin 8-O-β-D-glucopyranoside and chrysophanol 8-O-β-D-glucopyranoside have considerable activity in triton-induced rats by decreasing lipid level in plasma. The antidyslipidemic potential of ethanolic extract of *Rheum emodi* was reported by Mishra et al. (2014). *Rheum emodi* extracts have also exhibited the ability to mitigate a root-knot nematode, *Meloidogyne incognita* (Tripathi et al. 2014). A study found rhubarb to have anti-methanogenic activity on rumen fermentation and proposed that *Rheum* species should be taken into consideration for the production of feed additives to control methane production in ruminants (Arokiyaraj et al. 2019). Another research has shown the potential of rhubarb extract in extending the shelf life of beef (Cui et al. 2013). Rhubarb prevents diet-induced obesity and diabetes linked with increased *Akkermansia muciniphila* in mice (Régnier et al. 2020).

Tissue culture prospects

The plant, owing to its high medicinal and commercial value, has been extensively exploited over the past several decades. Different anthropogenic pressures like uncontrolled deforestation, selective extraction and un-sustainable harvesting by ethnic people also have pushed this species to the brink of extinction (Parveen and Wani 2013). Other reasons, such as poor seed germination and seedling survival in nature, have also contributed to a speedy decline in their population. The plant needs immediate attention for its sustainable utilization, cultivation, and conservation of the diminishing wild population of the species. Therefore, it has to be among the top priority species for conservation, multiplication and domestication to reduce pressure on natural habitats. Many countries have launched initiatives to support its commercial cultivation. For example, the National Medicinal Plant Board in India offers 50 per cent subsidy for its commercial cultivation. Apart from meeting the current demand, farming may conserve the wild genomic diversity of plant species. Similarly, in Ireland, its commercial cultivation on midland peatlands for fuel production has also attracted great interest as it yields not only a finished crop per hectare but produces more yields for biogas production also (https://biocapital.ie).

The development of agro-technology should be one of the thrust areas for research. To meet the growing demand for crude drugs, cultivation of the plant species is imperative. Plant tissue culture has the potential to address the problem in the cultivation of medicinal plants and play a significant role in the conservation of genetic material of many threatened species (Quadri et al. 2012); (Aquil et al. 2009; Shah, S. et al. 2013; Shah, S.N. et al. 2013; Xu, Y. et al. 2011; Xu, Y.W. et al. 2011a, b). In this technique, small parts of living tissue are isolated from an organism and grown on a nutrient medium under controlled conditions for indefinite periods under aseptic conditions (Dakah et al. 2014; Husaini and Abdin 2007; Husaini et al. 2011; Noor et al. 2022; Wasia et al. 2017). Embryo culture, organogenesis, and somatic embryogenesis methods have been used to regenerate plants through tissue culture (Husaini and Srivastava 2011; Shah, S. et al. 2013a; Shah, S.N. et al. 2013b; Wasia et al. 2017). Furthermore, several interactive biotic and abiotic factors have been examined for their effect on regeneration of various plant species (Bhat et al. 2016; Husaini and Abdin 2007; Husaini et al. 2011). The success of tissue culture depends largely on the explants' age, type and location; the most commonly used propagules are shoot tips, shoot buds and root tips (Sidhu 2010).

The tissue culture of *Rheum* has great potential. Many researchers have worked on in vitro multiplication of *Rheum australe*. Lal and Ahuja (1989) regenerated *Rheum emodi* from shoot-tip and leaf explants. It was observed that shoot-tip explants when cultured on medium with 2.0 mg/l BAP and 1.0 mg/l IBA gave rise to multiple shoots. Also, leaf explants on MS medium with 2.0 mg/l BAP and 0.25 to 1.0 mg/l IAA or IBA gave rise to shoot buds. Rooting was induced on MS medium with 1.0 mg/l IBA. *Rheum emodi* has been propagated using different liquid culture procedures and the best results were obtained using liquid shake cultures (Lal and Ahuja 1993). The direct appearance of shoot buds from the leaf surface without an intervening callus phase has also been reported by (Malik et al. 2009). In vitro propagation is affected by the concentration and combination of various growth hormones. The impact of varying BAP concentrations (2.5-15 μM) on shoot proliferation, individually and in combination with other auxins was assessed on the micro-propagated shoots of *R. emodi* cultured on MS medium (Parveen and Kamili 2012). Of all the treatments tested, 7.5 μM of BAP combined with 5 μM of IBA showed the best response for shoot proliferation. Further, the best rooting response was witnessed at 12.5 μM of IAA. Cytokinins encourage the development of leaves and new shoots in vitro (Salata and Kozak 2013). However, higher levels of BA (25 μM) along with 1.0 μM NAA were observed to induce direct somatic embryos in *R. spiciforme* (Khan et al. 2022). It has been shown that using a nitrogenous base (adenine sulfate) in conjunction with an amino acid (glutamine) is a more effective way to enhance the shoot multiplication process and preserve overall vigour (Siwach and Gill 2011). The cytokinin and auxin levels also control the initiation of callusing. (Singh and Chaturvedi 2022) used leaf and mid rib as explants and reported high callus initiation from mid rib (100%) and leaf (97.67%) explants. Further, leaf explants showed the highest shoot induction per cent of 76.25 and exhibited 70% rooting response. Callus proliferation from leaf, shoot tip and rhizome of other species of *Rheum* have also been reported. Callus formation was recorded from all the four explants (leaf, leaf mid rib, petiole and shoot tip explants) of *Rheum webbianum* at different concentrations and combinations of growth hormones after 4–6 weeks of initial inoculation (Tabin et al. 2018). The rhizome explant of *Rheum coreanum* exhibited successful callus induction on MS medium supplemented with 2.4-D (0.2–0.3 mg/l) (Mun and Mun 2016). The callus formed may be regenerative or non-regenerative in nature. High callusing on 2,4-D and BA (2 µM each) from leaf explants has been reported. However, the calluses obtained from leaf were mostly non-regenerative while the calluses obtained from nodes showed high regeneration on BA and kinetin (8.0 µM each) with 4.0 µM GA_3 (Khan et al. 2022). In another study, no regeneration was observed in callus obtained from seedling explants (Maithani 2015). For shoot regeneration, calli are sub cultured on MS media treated with various doses of BAP either alone or in combination with different auxins. In medium containing BAP (5 mg/l), the highest shoot induction from callus was seen after 10 days (Rashid et al. 2014). The main highlights of the in vitro studies are given in the Table 4. Although some micropropagation protocols of Rheum have been developed, various aspects of regeneration and micropropagation still need to be explored.

Conclusion

R. australe is an important medicinal plant used in different systems of medicine. Roots and rhizomes contain a variety of phytochemicals which exhibit various pharmacological activities like anti-cancerous, anti-oxidant, anti-bacterial, anti-fungal, anti-inflammatory and anti-ulcer activities. The plant has been overexploited for crude drug extraction from its natural habitats, rendering it endangered. Therefore, the preservation of the species' dwindling wild populace is necessary. Effective measures should be taken for sustainable utilization and for the development of agro techniques for cultivation in new habitats. Tissue culture has great potential and can be used for the conservation of germplasm of this endangered plant species.

Acknowledgements The authors are highly grateful to the Faculty of Forestry SKUAST-K and Division of Plant Biotechnology for the support and facilities.

Author contributions AH wrote the paper together with critical inputs from MA, THM and MAB. AMH and PRK conceptualized, analysed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest and nothing to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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Review

Challenges and Opportunities in the Sustainable Improvement of Carrot Production

Antonello Paparella [1], Prasada Rao Kongala [2], Annalisa Serio [1], Chiara Rossi [1], Liora Shaltiel-Harpaza [3,4], Amjad M. Husaini [5] and Mwafaq Ibdah [2,*]

[1] Department of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Via R. Balzarini 1, 64100 Teramo, Italy; apaparella@unite.it (A.P.); aserio@unite.it (A.S.); crossi@unite.it (C.R.)
[2] Newe Yaar Research Center, Agricultural Research Organization, Ramat Yishay 30095, Israel
[3] Migal Galilee Research Institute, P.O. Box 831, Kiryat Shmona 11016, Israel; liorat@migal.org.il
[4] Environmental Sciences Department, Faculty of Sciences and Technology, Tel Hai College, P.O. Box 831, Kiryat Shmona 11016, Israel
[5] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, SKUAST-K, Shalimar, Srinagar 19005, Jammu and Kashmir, India; amjadhusaini@skuastkashmir.ac.in
* Correspondence: mwafaq@volcani.agri.gov.il; Tel.: +972-4-9539537; Fax: +972-4-9836936

Abstract: From an agricultural perspective, carrots are a significant tap root vegetable crop in the *Apiaceae* family because of their nutritional value, health advantages, and economic importance. The edible part of a carrot, known as the storage root, contains various beneficial compounds, such as carotenoids, anthocyanins, dietary fiber, vitamins, and other nutrients. It has a crucial role in human nutrition as a significant vegetable and raw material in the nutraceutical, food, and pharmaceutical industries. The cultivation of carrot fields is susceptible to a wide range of biotic and abiotic hazards, which can significantly damage the plants' health and decrease yield and quality. Scientific research mostly focuses on important biotic stressors, including pests, such as nematodes and carrot flies, as well as diseases, such as cavity spots, crown or cottony rot, black rot, and leaf blight, caused by bacteria, fungi, and oomycetes. The emerging challenges in the field include gaining a comprehensive understanding of the interaction between hosts and pathogens in the carrot–pathogen system, identifying the elements that contribute to disease development, expanding knowledge of systemic treatments, exploring host resistance mechanisms, developing integrated control programs, and enhancing resistance through breeding approaches. In fact, the primary carrot-growing regions in tropical and subtropical climates are experiencing abiotic pressures, such as drought, salinity, and heat stress, which limit carrot production. This review provides an extensive, up-to-date overview of the literature on biotic and abiotic factors for enhanced and sustainable carrot production, considering the use of different technologies for the shelf-life extension of carrots. Therefore, it addresses the current issues in the carrot production chain, opening new perspectives for the exploration of carrots both as a food commodity and as a source of natural compounds.

Keywords: abiotic stress; agriculture; biotic stress; carrot; production

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Citation: Paparella, A.; Kongala, P.R.; Serio, A.; Rossi, C.; Shaltiel-Harpaza, L.; Husaini, A.M.; Ibdah, M. Challenges and Opportunities in the Sustainable Improvement of Carrot Production. *Plants* **2024**, *13*, 2092. https://doi.org/10.3390/plants13152092

Academic Editor: Nafees A. Khan

Received: 27 June 2024
Revised: 16 July 2024
Accepted: 23 July 2024
Published: 28 July 2024

1. Introduction

Food security and sustainable agriculture are crucial global priorities for human civilization and sustainable development. Biotic and abiotic stresses, as well as climate change, threaten agricultural production. For this reason, the development of sustainable solutions for agricultural production is urgent for the safety of both the planet and humans. In this regard, the carrots of the *Apiaceae* family and their yields in production areas are linked to biotic and abiotic threats that limit crop potential and associated industry.

Apiaceae is a family of vegetables and medicinal plants that holds 434 genera and nearly 3780 species [1], including many vegetable crops that are rich in flavonoids, carotenoids, coumarin, coumarin derivatives, vitamins, and minerals [2]. All over the world, carrots are among the top 10 vegetables for agricultural production [3]. The worldwide production of carrots and turnips reached 40.24 Mt from 1,082,967 ha in 2020 and about 41.67 Mt from 1,096,007 ha in 2021, with enormous horticultural and economic importance (FAO STAT, https://www.fao.org/faostat/en/#data/QCL, accessed on 26 June 2024). China, Russia, and the United States account for the main part, 34%, of global production [4]. The *Apiaceae* family includes carrots (*Daucus carota* L.), which are the most important *Apiaceae* cultivated worldwide. They originated in Middle Asia near Afghanistan [5,6] and gradually expanded into the Mediterranean region [7]. The earliest carrots were mainly purple or yellow, with some white or black species, instead of orange [8].

The cultivation of carrots involves various problems that arise at each stage of crop production and require attention and protection (Figure 1). These threats result in a reduction in crop yield and quality, which in turn leads to financial losses. Therefore, this review aims to provide a thorough analysis of the current scientific literature on carrot production, aiming to address challenges in the field and enhance the quality of carrots, offering new opportunities for utilizing carrots as a food commodity and as a source of bioactive compounds. This extensive analysis examines the wide range of biotic and abiotic stresses that can damage carrots, along with methods to control these factors. Moreover, different strategies to extend the shelf life of carrots during post-harvest are examined to promote improved and environmentally friendly production.

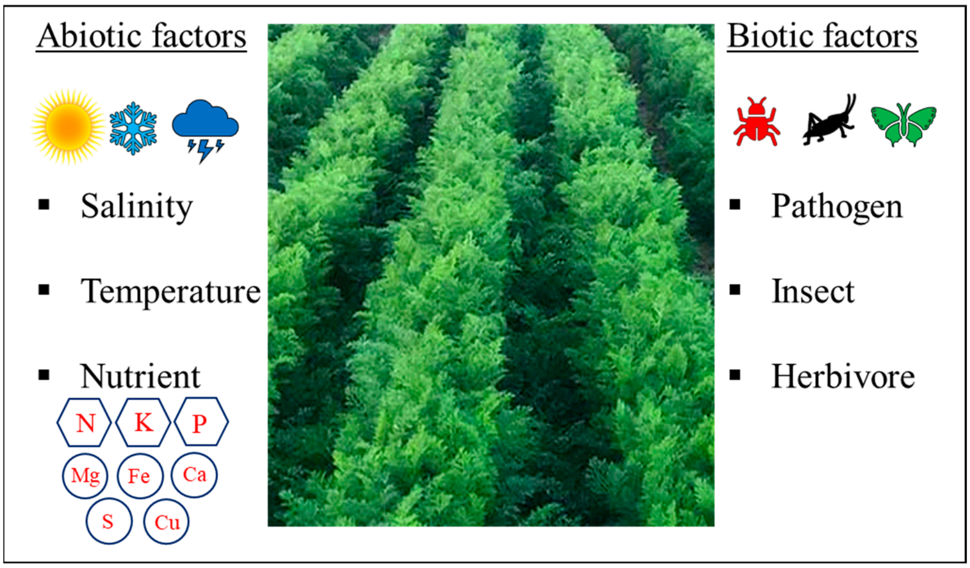


Figure 1. Photography of a carrot field depicting crops prone to abiotic and biotic factors affecting production and plant health (personal photos).

2. Bioactive Compounds Obtained from Carrots and Potential Applications

The species *D. carota* itself is a source of bioactive compounds that can be explored for different applications. The major phytochemicals in carrot roots are carotenoids (α- and β-carotenes, lutein, and lycopene), phenolic compounds (chlorogenic acid derivatives, caffeic acid, myricetin, luteolin, etc.), polyacetylenes (falcarinol and falcarindiol), and vitamins (vitamins C, E, K, B_1, and B_4; Figure 2), all considered as high-value bioactive molecules. Moreover, among fruits and vegetables, carrots are the richest source of β-carotene, which is the vitamin A precursor [9]. Carotenoids are great singlet-oxygen scavengers. Furthermore, diets rich in carotenoids, ascorbic acid, tocopherol, and non-vitamin antioxidants, such as anthocyanins and phenolic compounds, contribute to protecting DNA and proteins from oxidative processes [10]. Noticeably, black or purple carrots are particularly rich in acylated anthocyanins, which exert high antioxidant activity and nutraceutical features [11].

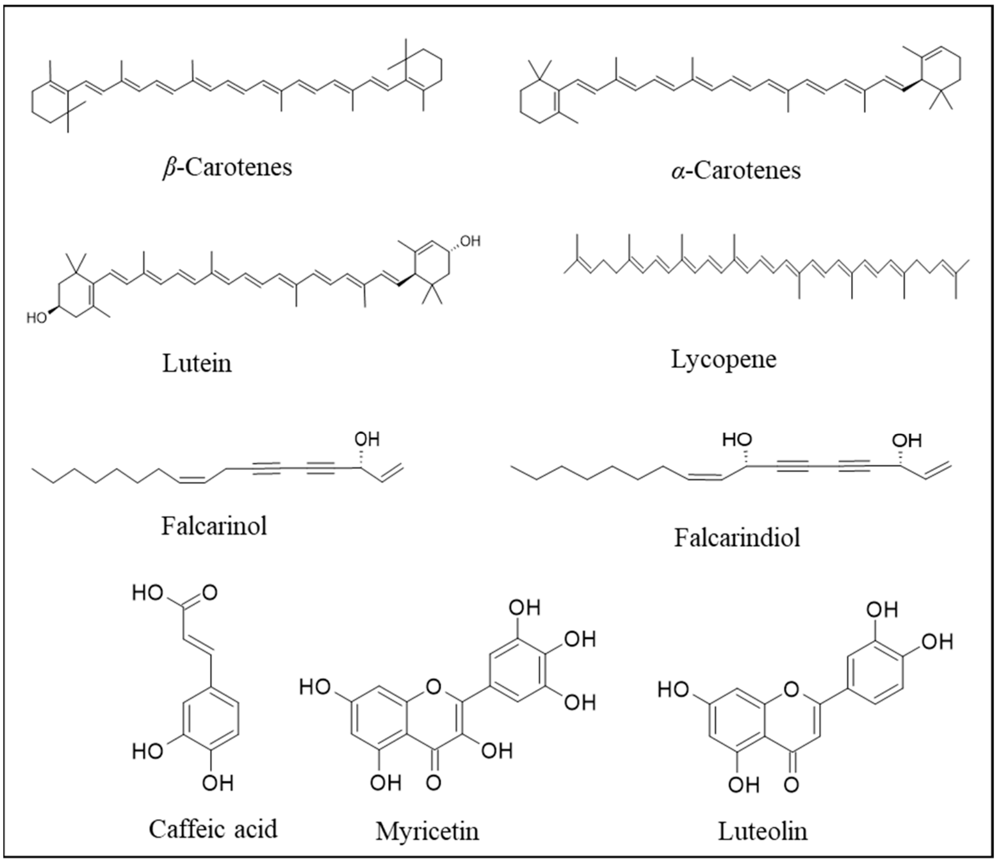


Figure 2. Carrot-root-sourced prominent nutraceuticals and other significant metabolites.

Carrot seeds are rich in petroselinic, linoleic, and palmitic acids, proteins, and fibers, and from them, both oil and essential oil can be extracted (Figure 3). Carotol is the main component of both seed edible oil (30.55%) and seed essential oil (66.78%) [12]. Traditional medicine suggests carrot seed EO as a tonic to treat digestive problems.

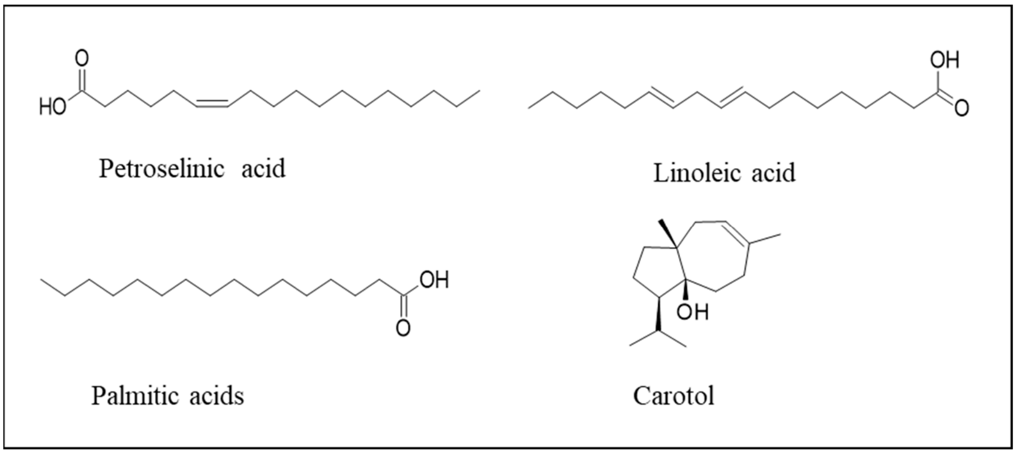


Figure 3. Major bioactive compounds obtained from carrot seed essential oil.

This EO also has analgesic, anti-inflammatory, antimicrobial, and antioxidant activity [13], and it is generally regarded as safe when ingested in low amounts as food flavoring. Carrot seed EO is the main source of carotol used in cosmetics for fragrance synthesis. Depending on the chemotype, this EO shows moderate and non-specific toxicity on different cell lines [13].

The EO can also be extracted from the dried fruit. Also, in this case, the most important compounds are sesquiterpenic molecules, such as *β*-caryophyllene, and the alcohols, carotol and daucol. Carrot EO exerts antimicrobial activity on bacteria and fungi, with a greater effect on Gram-positive bacteria, as it is usually observed for other EOs. Also, an extract obtained by supercritical fluid extraction (SFE) by means of carbon dioxide had carotol as the main compound (30.3%), and good quantities of geranyl acetate (7.22%), *β*-caryophyllene (6.47%), and daucol (2.46%).

Still, it did not contain lighter components present in the EO, such as *α*-thujene, *α*-pinene, *β*-myrcene, *γ*-terpinene, *o*-cymene, and linalool (Figure 4). EO and SFE showed antimicrobial activity against Gram-positive bacteria, such as *Bacillus subtilis*, *B. cereus*, and *Rhodococcus equi* [14]. Carotol itself exerts fungicidal activity on *Alternaria alternata.*

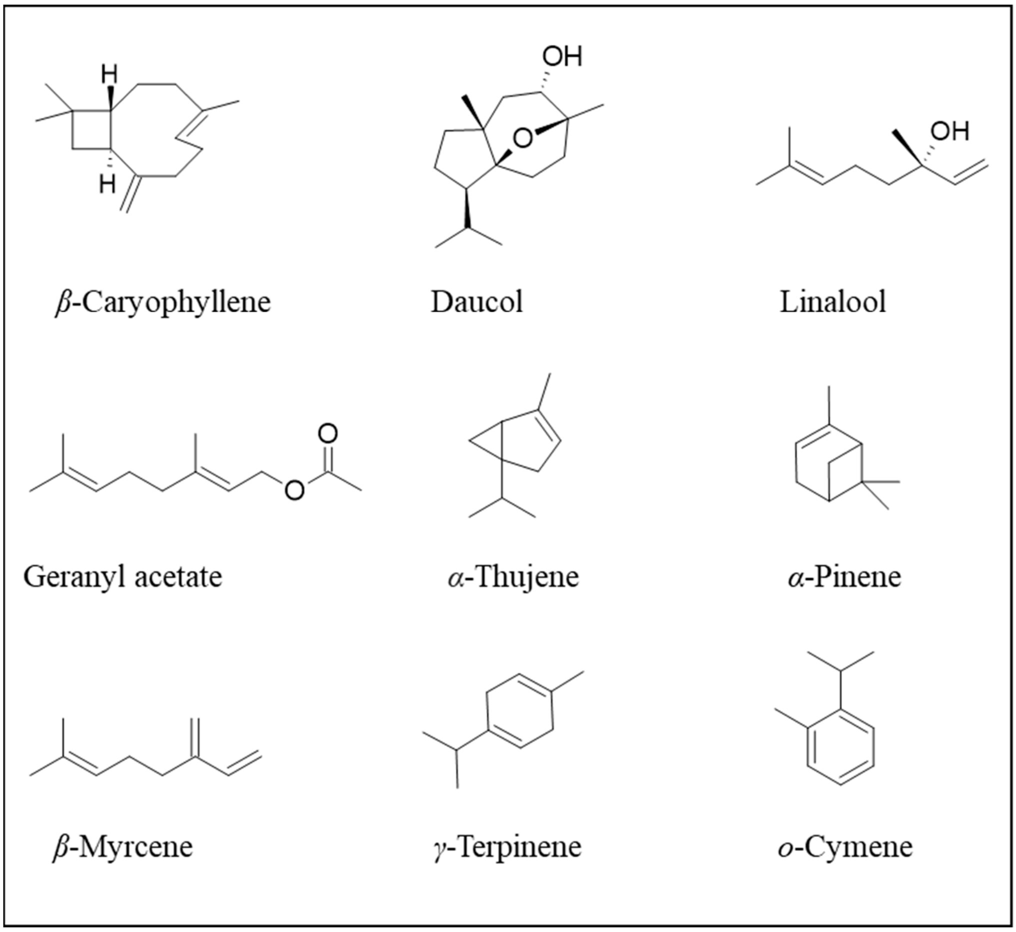


Figure 4. Major volatile organic compounds found in the carrot essential oil with different biological activities.

An EO extracted from carrot umbels showed minimal inhibitory concentrations of 0.32–0.64 µL/mL against *Bacillus subtilis, Staphylococcus aureus*, and *Listeria monocytogenes*, and was also effective on dermatophyte strains and *Cryptococcus neoformans* (MIC of 0.16 µL/mL). In particular, the EO inhibited germ tube formation and filamentation (essential for virulence) in *Candida albicans* at very low concentrations, decreasing biofilm mass and cell viability [15]. As biofilm is difficult to prevent and eradicate, these results could be useful for implementing strategies to counteract candidiasis. The same EO demonstrated anti-inflammatory activity, decreasing the production of nitric oxide (NO), which is a mediator in the inflammatory response, in LPS-stimulated macrophages at concentrations safe for macrophages, hepatocytes, and epithelial cells [15].

Interestingly, a commercial wild carrot EO, rich in methyl isoeugenol (60.7%), and an EO extracted from umbels of wild carrots collected in Illinois had a toxic effect on mosquito larvae, such as *Aedes aegypti*, *Culex pipiens* L., and *Culex restuans* Theobald, therefore suggesting an application as a biopesticide [16]. In addition, a carrot seed EO, rich in carotol (>70% w/w), showed high repellency against *Aedes aegypti* and *Anopheles quadrimaculatus*. Even carotol alone showed a biting deterrent activity similar to diet in laboratory studies. Moreover, both carotol and carrot seed EO showed good repellency in skin application assays, showing the potential to be explored as a natural repellent in commercial formulations [17].

Moreover, different medical applications for EOs and solvent extracts have been proposed, and in detail, carrot bioactive compounds, such as lutein, beta-carotene, and polyacetylenes, as well as EO, were demonstrated to possess anticancer activity against different carcinoma and leukemia cell lines, ethanolic extracts rich in flavonoids and phenolic derivatives accelerate the wound healing process, while coumarin glycosides lower blood pressure and relax muscles. Carrot extract has been proven to have gastroprotective activity, thus supporting the traditional use in gastric ulcer and acidity treatment [18]. Carrot seed EO is used as a tonic and stimulant for skin problems and to treat hepatic and renal insufficiency [14]. On the other hand, it needs to be taken into account that bioactive polyacetylene compounds negatively impact the taste of carrot roots because they increase bitterness [19].

Interestingly, bioactive compounds are also contained in carrot waste and, therefore, food industries and research partners are focusing their activity on the recovery and valorization of these molecules from waste to enrich the nutritional profile of food products and beverages [10].

3. Biotic Threats: Carrot Diseases

The carrot crop is damaged or infected by over 150 species of insects, mites, nematodes, fungi, viruses, bacteria, or phytoplasmas. Among these, pests, such as nematodes, carrot flies, and diseases, including cavity spots, crown or cottony rot, black rot, or leaf blight, emerged as major challenges worldwide [20].

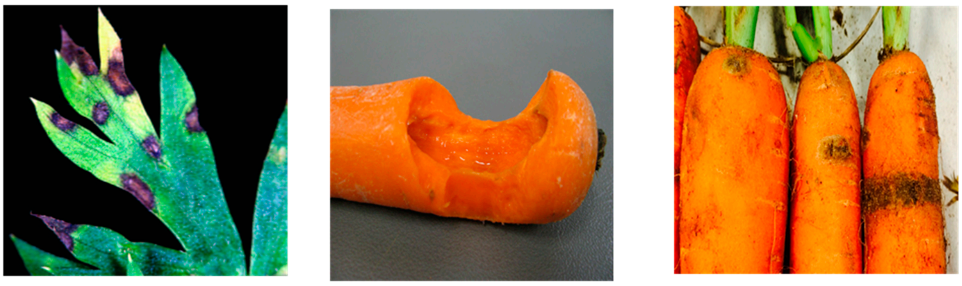
The interaction between carrots and pathogens, which leads to the development of disease, is influenced by various factors, including agroecological parameters, host specificity, growing stage, phytotoxic compounds, and the virulence of the strain. A comprehensive comprehension of disease progression, together with the analysis of genetic and observable traits, will facilitate the identification and cultivation of superior carrot cultivars. The management tactics are always changing based on the specific characteristics of the carrot–pathogen interaction and the roles of the geographical agro-climate system in crop production.

3.1. Bacterial Diseases

3.1.1. Bacterial Leaf Blight

Xanthomonas hortorum pv. *carotae*

Xanthomonas hortorum pv. *carotae* (*Xhc*) causes carrot bacterial leaf blight, which can be spread through seeds [21]. Carrot fields throughout Europe, North America, and Asia frequently show signs of its presence [22]. According to Pruvost et al. [23], the seed serves as a primary source of inoculum. The bacteria can persist in carrot remnants and can be transmitted through carrot seeds, but they are unable to thrive in the soil in the absence of debris. Temperatures ranging from 25 to 30 °C (77° to 86 °F) are conducive to the spread of infection and the development of diseases (Figure 5). The bacteria are dispersed through the action of water splashing, and plant-to-plant dispersal can occur under times of heavy dew. Control measures for bacterial blight are often unnecessary in the majority of regions where carrots are grown. To achieve optimal disease growth, either rain or spray watering is required.



Bacterial leaf blight Bacterial soft rot Carrot scab

Hairy root Crown gall

Figure 5. Pictures of carrot leaves and roots depicting bacterial diseases and symptoms (personal photos).

Within carrot fields, these brown spots are frequently identified as the initial signs of the disease, which are then followed by small, irregular yellow lesions on the leaves, stems, and petioles. These lesions may resemble water-soaked necrotic lesions [24]. The leaf exhibits irregular brown patches, typically originating from the edges of the leaf. Lesions first exhibit an asymmetrical golden halo and may appear saturated with water. Spots merge together and result in leaf disease, while leaf petioles develop dark brown streaks. Floral components might also experience blight.

An adhesive, amber-colored bacterial secretion (a diagnostic sign of the disease) could be seen on foliage or trickling down on leaf stems and flower stalks. The acceptable methods of control include: (i) cultural controls, (ii) the use of *Xanthomonas*-indexed seed or treated seed in hot water dips, (iii) the application of certain copper sulfate formulations through spraying, (iv) furrow or drip irrigation instead of sprinklers, (v) burying leftover carrot scraps to accelerate the process of decay, and (vi) preventing the persistent cultivation of carrots by implementing a crop rotation plan that spans two to three years.

3.1.2. Bacterial Soft Rot

Klebsiella variicola
Pectobacterium spp.
Dickeya dadantii

Bacterial soft rot by *Klebsiella variicola* is a major constraint in carrot farms. It is one of the most devastating diseases [25]. Most bacterial soft rot agents are members of *Pectobacterium* spp. and *Dickeya*, with the former genus encompassing a constantly growing number of species varying in geographic distribution and host of isolation [26]. Carrots with severe soft rot symptoms on the tap root (Figure 5) have a putrid smell, wilting, and foliage collapse [25]. Biological agents, specifically strains A6 and P42 of *Bacillus velezensis*, have been found to be effective in managing soft rot disease in carrots. These strains have shown antagonistic properties against *K. variicola*, making them suitable biocontrol agents. This approach is considered more environmentally sustainable compared to the use of agrochemicals.

3.1.3. Hairy Root

Agrobacterium rhizogenes

Hairy root production in carrots is caused by the infection of *Agrobacterium rhizogenes*, resulting in the development of proliferative multi-branched adventitious roots at the site of infection [27]. Carrots grown in soils that are moderately dense and contain a high amount of decomposed organic matter often exhibit an overabundance of leaves (Figure 5) and generate roots that are hairy and forked. Additionally, the outer texture of these carrots tends to be rougher and coarser. Hairy roots serve as a biological platform for synthesizing a wide range of complex biomolecules.

3.1.4. Crown Gall

Agrobacterium tumefaciens

In carrots, crown gall is caused by *Agrobacterium tumefaciens* (Sm. and Town.), which can produce galls as a result of residing in a tumor-inducing (Ti) plasmid. The carrot crown gall manifests as tubular to irregular, yellow to tan galls located on the stem in close proximity to the crown or on the roots. Galls typically form at the junction of lateral roots and the tap root. Nevertheless, galls can form in any location where the plant has sustained damage (Figure 5). Multiple galls, varying in size, can develop on a plant during midsummer and continue growing in quantity and size until harvest. To address this issue, implementing cultural methods, such as employing extended crop rotations with onion, maize, oats, grasses, and other resistant crops, can potentially lead to a decrease in soil bacterial populations. Biological management of crown gall on stone fruits and roses has been successfully accomplished by introducing a harmless strain of *Agrobacterium radiobacter* (Beij. & Van Delden) Conn into these plants. This method has been documented by several studies [28–30].

3.1.5. Scab

Streptomyces scabiei

Scab disease of carrots is caused by three different bacteria: *Streptomyces acidiscabies*, *S. caviscabies*, and *S. scabiei*. These bacterial diseases affect the marketable production of carrots in both field and greenhouse settings [31–33]. Only a small number of *S. scabies* infections result in damping-off. Plants affected by scabies exhibit characteristic scab symptoms on their roots, where scab lesions are generated due to the aberrant growth of the host cells. This leads to the formation of corky tissue that is typically darker than healthy tissue (Figure 5). Lesions can occasionally be either depressed below or elevated above the level of the intact skin. Multiple individual lesions have the potential to merge together, resulting in the formation of contiguous scabby regions.

The severity of the scab is rarely significant enough to necessitate particular control techniques. Nevertheless, it is worth noting that alkaline soils tend to promote the growth of scab in other crops, including potatoes. Therefore, to mitigate the disease, it is advisable to refrain from cultivating carrots in alkaline soils or to use fertilizers that have an acidic effect, such as ammonium sulfate or sulfur, to decrease the pH of the soil. Cultivating carrots in soils that have a high capacity to retain moisture or implementing irrigation practices to ensure a consistent water supply can potentially decrease the occurrence of scab disease. Moreover, it is advisable for farmers to refrain from cultivating carrots on fields that have been previously used for potato farming. Implementing extended crop rotations involving small grains, grasses, or maize can potentially decrease the severity of scabs [31,34,35]. Thaxtomin A, a phytotoxin produced by *Streptomyces* spp., is the primary virulence determinant of scab in carrots. Due to the shared characteristics between potatoes and tap root crops in terms of infecting strains and the key virulence factor, thaxtomin A, it is advisable to evaluate the effectiveness of management methods developed for potatoes in controlling *Streptomyces* scab disease in carrots [36].

3.2. Fungal and Oomycete Diseases

3.2.1. Alternaria Leaf Blights (ALB)

Alternaria dauci (J. G. Kühn)

Alternaria leaf blight (ALB) of carrots, caused by the fungus *A. dauci* [2], is one of the most common and destructive diseases worldwide [37]. Typically, the *A. dauci* infection leads to extensive deterioration of the leaves and main root, causing substantial reductions in crop productivity [38]. In the beginning, the infection affects the foliage and petioles, with small areas of dead tissue with varying forms and sizes (Figure 6) [33,39]. The spots gradually expand and merge until the entire leaf withers. Hence, the process of mechanical harvesting becomes challenging, ultimately resulting in a substantial decrease in crop production [5,6]. During epidemics, crop output reduction can reach 90% [40,41].

Figure 6. Photographs of carrot leaves and roots illustrating fungal and oomycete diseases and symptoms (personal photos).

Investigating the resistance mechanisms of carrots to the leaf metabolites produced by *A. dauci* could be a promising avenue of research. The major leaf compounds found against *A. dauci* of carrot varieties and accessions were terpenes, such as myrcene, sabinene, *trans*-α-ocimene, limonene, germacrene D, *trans*-α-caryophyllene, β-caryophyllene, β-myrcene, and α-pinene [4,42,43].

The elucidation of the biosynthetic pathway for luteolin and apigenin derivatives, which are flavonoids, will provide a crucial foundation for conducting functional and genetic studies of flavone production in carrots. A study conducted by Koutouan et al. [44] demonstrated that the growth of *A. dauci* conidia was hindered by two secondary metabolites found in carrots, namely, falcarindiol and 6-methoxymellein. The cultivars that are resistant and susceptible to *A. dauci* showed varying levels of accumulation of falcarindiol in their leaves, suggesting that falcarindiol plays a role in resistance to *A. dauci* [45]. Currently, ALB stands as the most detrimental foliar disease. No known resistance gene can effectively combat this fungus [39]. At present, all resistant cultivars only possess partial resistance, thus requiring the continued use of fungicide treatments.

3.2.2. Black Root Rot (Black Mold)

Trichocladium basicola (Berk and Broome)

Black root rot is a highly destructive disease that affects fresh carrots growing in muck soils after they have been harvested. Lesions consistently develop in the locations where wounds are acquired during the processes of harvesting, grading, and sorting. The symptoms consist of superficial black lesions that are irregularly and randomly distributed (Figure 6). These lesions range in size from 3 to 20 mm. These formations occur under high relative humidity conditions on roots that have been washed, sorted, and stored in polyethylene bags at temperatures exceeding 25 °C. Only the epidermis is affected by root discoloration caused by widespread fungal sporulation.

The disease occurrence is linked to the storage of recently harvested carrot roots at elevated temperatures and relative humidity. Storing carrots at the ideal temperature of 0 to 1 °C and relative humidity of 98 to 100% rarely poses a significant issue. Before grading, it is advisable to eliminate as much soil that is clinging to the roots as feasible. Moreover, refrigerating just-gathered carrots is recommended. The storage temperature must be kept below 7 °C. Carrots should be subjected to chemical control by immersing them in chlorinated water prior to being packed in plastic bags [46–48].

3.2.3. Black Rot

Alternaria radicina

Black rot is caused by the infection of *Alternaria radicina*, leading to the formation of black spots. The markings on the leaves and leaf stem are similar to those caused by *A. dauci*, characterized by irregular black lesions, especially along the margins of mature leaves. The infection can infiltrate the vascular bundle on the petioles, resulting in the leaf undergoing a yellowing process, wilting, and ultimately perishing (Figure 6). The size and pattern of the spots can range from tiny linear lines to very large round patches. Infections induced by *A. radicina* are generally less severe compared to those caused by *A. dauci*. *A. radicina* grows within a temperature range of −0.5 to +34 °C, specifically when the moisture level of the air reaches 92%. The disease primarily affects carrots during storage [39,49]. Infected carrots can also contaminate any nearby healthy carrots, serving as a source of infection.

A. radicina can be found on several parts of the plants, including seeds, umbels, foliage, petioles, and roots. On seedlings, it causes seed decay, damping-off, blackened hypocotyls, and malformed roots. On seeds, it forms a diffuse black weft of mycelium, which can cover the seeds and include black conidia. Additionally, the bottom portion of the tap root is destroyed. Seed-borne infection or planting in infested soil may lead to pre- and post-emergence damping-off. Affected seedlings have tan-brown to black lesions constricting the stem, which may be continuous. This lesion can grow from the soil level upwards and sometimes reach the cotyledons. Growers must implement at least eight years of crop rotation with crops other than carrot, dill, parsley, parsnip, and celery, using only seeds treated with hot water or a fungicide. In addition, crop debris should be removed immediately. For optimal preservation, it is recommended to maintain a storage temperature of approximately 0 °C and a humidity level of around 92% to minimize deterioration. The use of fungicides for chemical management can effectively decrease the occurrence of storage decay during the foliar phase [50].

3.2.4. Crown Rot (Rhizoctonia Canker)

Rhizoctonia solani Kühn, Anamorph of *Thanatephorus cucumeris*

Crown rot by *Rhizoctonia solani* also causes damping-off of carrot seedlings, usually more damaging on the roots of larger carrots, which results in a significant decrease in crop productivity [51–53]. The fungus has an extensive host range among vegetable crops [51,52,54]. Prolonged mid-season infections cause rot during storage. The earliest marks of crown rot are horizontal dark-brown lesions that form at the locations where lateral roots emerge from the tap root. These lesions may extend a few millimeters into the tap root, differently from cavity spot lesions caused by *Pythium* spp. The lesions of crown rot are numerous on the upper portion of the root. The disease is also characterized by the presence of a dark brown, dry rot that forms a band around the crown. The external foliage of the impacted roots withers and perishes, resulting in the plant having a limited number of larger internal leaves that remain erect [51–53]. Upon extracting the diseased roots, substantial amounts of dirt and mycelium can be found.

Carrot crown rot is a significant disease that severely limits or decreases the number of marketable carrots and overall profitability. The carrot crown displays several symptoms that impact its marketability, including ring crown rot, smooth crown rot, corky crown rot, soft watery crown rot, and black ring (Figure 6). Carrots that are infected will exhibit lesions on their roots, which can harm the overall health of the crop and result in lower grades for the saleable produce. With the exception of black rings, all other faults in carrots are considered significant and will result in the carrots being discarded as trash. On the other hand, carrots with black rings on the tops are classified as lower-grade and are sold for less than half the price of premium-grade carrots.

The most severe occurrence of crown rot and damping-off is observed at temperatures ranging from 20 to 28 °C, while infection or disease development is minimal below 16 °C. Optimal disease development occurs when soil moisture levels are above field capacity, which is about -0.1 bar [51,52]. The diverse soil factors and field conditions seem to have a huge influence on the types of crown rot symptoms, including (i) soil compaction caused by prolonged wet conditions and soil crusting, which appears to be a major contributing factor to crown rots, (ii) early rubbing friction in dry soil crust and other physical injuries that precede the development of ring crown rots, and (iii) infections in tall, dense crop canopy, such as *Sclerotinia* and other foliar diseases, causing soft watery crown rot in cool, wet conditions.

Carrot plants of all ages are equally vulnerable to *R. solani*, with crown rot being more severe in older plants. To decrease the occurrence of damping-off in carrot seeds, it is recommended to apply a fungicide to the seeds, as suggested in [53,55–57]. Moreover, the risk of crown rot should be reduced by treating the soil surface after cultivation to break up any crust that has formed on the top layer. In addition, carrot tops are encased in soil to shield them from drastic changes in moisture and temperature levels on the surface of the soil. Finally, trimming carrot tops either horizontally or vertically may potentially help reduce the occurrence of soft, watery, and black ring crown rot in carrots.

3.2.5. Ring Rot Disease (Pythium Root Dieback)

Pythium coloratum (Vaartaja)
Pythium irregulare (Buisman)
Pythium sulcatum (Pratt & Mitchell)
Pythium sylvaticum (W.A. Campbell & J.W. Hendrix)
Pythium ultimum (Trow)

Pythium sulcatum, a soil-borne pathogenic oomycete that morphologically resembles a fungus, is responsible for the highly damaging cavity spot disease [7,58]. The characteristic symptoms consist of concave, round-to-elliptical lesions 2 to 5 mm-long [59]. The tap root may be branched and surrounded by several elongated lateral roots. In other cases, the size may be greater, but the growth is stunted or divided into multiple branches. The foliage often appears robust, although occasionally, it may appear stunted or wilted. Severely affected seedlings may wilt and die [58]. Mature plants have the potential to recuperate by developing a large number of side roots, but these plants usually yield tap roots of inferior quality. The disease has also been referred to as rusty root, lateral root dieback, and forked root [7,58].

The hyphae of *Pythium* spp. are hyaline and aseptate, except for old hyphae. Septa are found at the base of reproductive structures. Young hyphae exhibit cytoplasmic streaming, as observed by Van der Plaats-Niterink [60,61]. According to Howard et al. [62], carrot plants that were cultivated in sand contaminated with *P. ultimum* and kept at a soil moisture potential of −2.5 kPa exhibited a higher number of forked roots at a temperature of 23 °C, compared to 27 °C. *P. ultimum*, *P. aphanidermatum*, and *P. irregular* exhibit increased lethality toward carrot seedlings when exposed to a temperature of 35 °C instead of 25 °C.

The implementation of cultural techniques, including the cultivation of carrots on raised beds, has been found to effectively decrease the occurrence of root forking and improve the percentage of marketable carrots. Carrots should not be cultivated in fields with inadequate drainage or susceptible to flooding. Additionally, it has been demonstrated that precision seeding effectively decreases the occurrence of root dieback. Implementing crop rotations with cabbage, corn, mint, onion, and potato has the potential to decrease the occurrence of *Pythium* root dieback in subsequent carrot crops. Finally, commercial cultivars should possess a high level of tolerance to *Pythium* root dieback [63].

3.2.6. Cavity Spot

Pythium intermedium (de Bary)
Pythium irregulare (Buisman)
Pythium sulcatum (Pratt & Mitchell)
Pythium sylvaticum (W.A. Campbell & J.W. Hendrix)
Pythium ultimum (Trow)
Pythium violae (Chesters & C.J. Hickman)

Cavity spots are caused by various *Pythium* species, including *P. violae*. Carrots infected by *Pythium* spp. show symptoms of root dieback and have numerous rusty-brown lateral roots [64]. Carrots planted in recently cleared land or cultivated fields where umbelliferous crops have never been grown may develop severe cavity spots.

Conversely, fields where carrots have been cultivated repeatedly may have no history of cavity spots. Fields known to produce carrots infected with cavity spots may not show disease from one year to the next, depending on environmental conditions. First, symptoms appear under intact periderm as sunken areas that are either gray or not discolored [65]. The cavities resemble elliptical lesions that are sunken a few millimeters below the root surface. The lesions are elongated horizontally, arranged randomly, and darkened with age.

Lesions vary in size, and secondary organisms may infect the carrot, causing rapid rotting. The size of the cavities expands proportionally with the growth of the roots. Vertical cracks are sometimes associated with the cavities. Regarding cultural methods, carrots on raised beds are used to reduce the likelihood of excessive soil moisture levels and avoid using fields with a history of cavity spots. The utilization of resistant cultivars will facilitate progress. The severity of cavity spots has been linked to the use of high rates of chemical fertilizers and to increases in soil moisture either early in the season or throughout maturation. Simultaneously, decreases are found in soils with a pH higher than 8 [66]. Carrots with cavities are not suitable for sale in their fresh state or for processing, and their overall yield can be significantly diminished.

3.3. Insects

3.3.1. Carrot Psyllids

Candidatus Liberibacter solanacearum

Carrot psyllids (*Trioza apicalis*, *Trioza anthrisci*, *Bactericera nigricornis*, and *B. trigonica*) are the insect vectors that feed on the carrot leaves, causing substantial damage to the growing crop. Moreover, the psyllids can transmit a bacterial pathogen called *Ca. L. solanacearum*, a vector-transmitted yet-unculturable alpha-proteobacterium associated with carrot diseases [67].

Ca. L. solanacearum has ten divergent haplotypes identified, which cause different diseases in host plants over a wide geographic distribution. The haplotypes C, D, and E cause diseases in carrots and celery in Europe [68]. The haplotype C is transmitted by *T. apicalis* Forster in northern Europe [69], whereas the haplotypes D and E are transmitted by *B. trigonica* Hodkinson in the Mediterranean area on both carrot and celery. The recently identified haplotype H was found to infect carrots and parsnips (Figure 7).

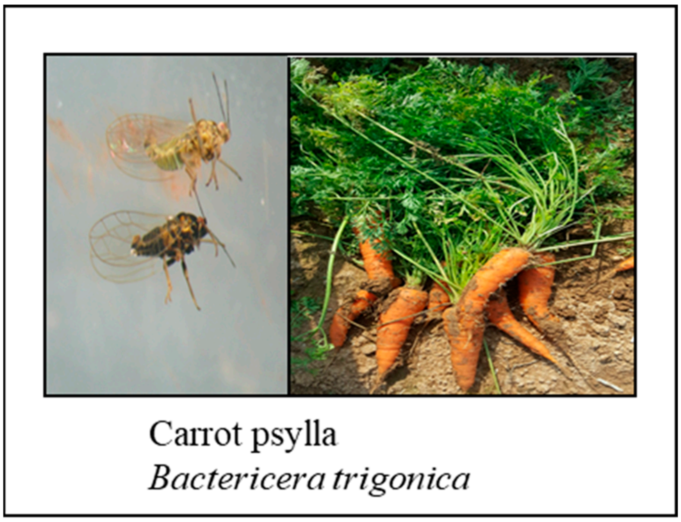


Figure 7. Photographs of carrot psylla and carrot symptoms (personal photos).

Carrot psyllid *T. apicalis* feeding exhibits typical symptoms of leaf curling and stunted growth of the shoot and root, whereas the symptoms associated with phloem limited pathogen *Ca. L. solanacearum* haplotype C bacterial infection, which causes leaf discoloration, and reduced the storage root weight [69]. Leaf curling is a rapid response to psyllid, but the *Ca. L. solanacearum* symptoms develop slowly and become visible 1.5 months after inoculation when the bacterial titer is high [69,70].

The shoot proliferation symptoms (i.e., witches' broom) in carrots are caused by *Candidatus* Phytoplasma and *Spiroplasma* infection. The *Ca. L. solanacearum* haplotype-D-associated shoot proliferation symptoms are influenced by temperature, plant age, and vector load, being very sensitive at 30 °C and favored at 18 °C [71].

3.4. Microbial Ecology of Carrots

Vegetables, including carrots, can be contaminated by both spoiling and pathogenic microorganisms directly via the seeds or during cultivation, harvesting, post-harvesting procedures, processing, and storage, up to the distribution [72]. In particular, the microbial ecology of carrots is strictly related to the quality of the soil where they are cultivated. Moreover, raw or improperly composted manure, as well as low-quality water used for irrigation, may be an important source of microorganisms, including pathogens and antibiotic-resistant bacteria. Among the bacteria, Dharmarha et al. [73] reported the presence of *Gammaproteobacteria, Bacilli, Betaproteobacteria, Actinobacteria,* and *Alphaproteobacteria,* from the most to the least abundant, for a total of 114 different families, with 78% of bacteria belonging to the families *Pseudomonadaceae*, *Enterobacteriaceae*, *Oxalobacteriaceae*, *Bacillaceae*, and *Paenibacillaceae*. Although these families are common on other vegetables, it has to be underlined that *Enterobacteriaceae* also include pathogenic bacteria. For example, the presence of *Yersinia pseudotuberculosis* was associated with gastrointestinal disease in 2004 and 2006 in Finland [74,75]. Also, *Salmonella* spp. has been related to different vegetables, including carrots [76]. As regards non-pathogenic bacteria, the presence of psychotropic *Pseudomonas* spp., coliforms, and *Enterobacter* spp. is reported on fresh carrots and normally increases during refrigerated storage [72]. The total aerobic count can be as high as 7.9 Log CFU/g on whole carrots [72]. Also, *Dickeya, Pectobacterium* (both previously belonging to the genus *Erwinia*), and *Pseudomonas* are commonly reported in carrots, although not all *Pseudomonas* strains are responsible for spoilage. In addition, yeasts and molds can be recovered on carrots, deriving from in-field contamination, and their counts often arise during storage.

Spoilage

Carrots are among the most consumed vegetables worldwide—they are cultivated root vegetables, often stored for long times for year-round supplies, as in Northern Europe. Nevertheless, long-term storage impairs the nutritional and microbiological quality of the product [77]. Carrots contain about 90% of water, with 7.6% available carbohydrates and deficient amounts of lipids (0.2%) and proteins (1.1%) [78]. Considering the low fat and protein content, it can be inferred that much water is available for microbial development. Additionally, polysaccharides are converted into simple sugars, easily employable by microorganisms to sustain their growth during storage.

Carrots are exposed to colonization by fungi and bacteria already during their cultivation. In particular, when soil conditions are wet, spoilage is favored [79]. The same microorganisms can also be recovered from the fresh product. Microorganisms first grow on the surface of the vegetable, but some of them possess lytic enzymes, such as pectolytic and cellulolytic enzymes, allowing them entrance into inner tissues. The process is facilitated with fresh-cut carrots. The most common changes due to microbial growth are weight loss, bitterness, bacterial deterioration, and sprouting. Moreover, carrots quickly lose firmness while off-odors develop as a consequence of the high respiration rate and microbial growth [80]. The most common spoilage bacteria occurring on fresh, unprocessed carrots are those belonging to the genera *Dickeya*, *Pectobacterium*, and *Pseudomonas.* The first two are able to colonize carrots first in the field, where they can cause plant disease, and then post-harvest during storage, while *Pseudomonas* is mainly responsible for post-harvest spoilage. *P. viridiflava, fluorescens, cichorii*, and *marginalis*, as well as *P. carotovorum* subsp. *carotovorum* and *D. chrisanthemi* cause carrots to soft rot [77]. Particularly, *P. carotovorum* subsp. *carotovorum* can cause significant losses if left uncontrolled. Spoilage generally starts from the crown or root tip and continues rapidly toward the innermost region [81]. Apparently, the peel and the color of the carrot remain intact, while the root becomes watery, slimy, and soft, with a rotten odor. Moreover, secondary fungi often grow in rotten areas. Also*, Erwinia rhapontici* has been reported to cause carrot spoilage and is associated with cavity formation [77]. As already described, different fungi are related to plant diseases in the field. Nevertheless, some are also responsible for spoilage during storage in refrigerated or room-temperature conditions. In detail, *Botrytis cinerea* causes black lesions, where the production of grey spores can be noticed. When carrots are stored at room temperature, black root rot can occur. Moreover, *Chalaropsis thielavioides* and *Thielaviopsis basicola* are responsible for the black spots on the surface of the carrot, which can be covered entirely in a few days, making the vegetable unsuitable for consumption. Washing and refrigerated storage can help control microbial growth. Based on post-harvest storage methods, unprocessed carrots' shelf life can vary from one week to one year or more.

4. Influence of Abiotic Stresses on Carrot

In vegetable crops, growth, development, and yield are affected by abiotic stresses, such as soil salinization, low and high temperatures, and drought. To overcome stresses and survive, crop plants evolve different protective mechanisms [82]. Crop plant improvement for high yield and tolerance to abiotic stresses by breeding are effective strategies, leading to sustainable agricultural production and safeguarding food supplies [83]. Investigating the physiological mechanisms and their regulation is important for the development of stress-tolerant plants using either conventional systems or bio-technological approaches [84,85]. Carrots are categorized as an excellent crop since they do not demand the warmer conditions required to produce vegetables, as tomatoes or cucumbers do. An optimal growing temperature of 17 °C with a range from 7 to 24 °C is typical [86] for carrot production in temperate climates. However, relatively little has been reported for high-temperature effects on carrot growth.

Abiotic stresses influence the changes in phenolic compounds in carrots. Oliveira et al. [87] observed that the activity of the phenylalanine ammonia-lyase (PAL) significantly increased with subsequent increments of 1000–1500% of total phenolic content after 72 h at

15 °C in wounding and moderate UV-C pretreatment. Hyperoxia storage even improved total phenolic increments by up to 2000%, partly profited by mild water stress. UV-C pretreatment has reduced PAL activity, favored by a higher electrolyte leakage. Post-harvest abiotic stresses resulting in phenolic accumulation of carrots leading to greater assimilation of antioxidant compounds can be used to increase the health-promoting properties of carrots, at the same time meeting food safety requirements related to the use of a moderate UV-C dose.

4.1. Salinity Stress

Carrots, as a glycophyte root crop, exhibit sensitivity to salinity [88]. They conveniently grow in soils containing low-sodium salts. The carrot accessions exhibit varied responses to salinity, and highly saline-growing carrots are also reported [89,90]. Under salt stress, carrot plants' responses are inhibition of growth, abnormalities in morphological characters, and accumulation of malondialdehydes (MDA) membrane lipid degradation products. Biochemical activity reveals reduced soluble protein content and lower superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activity. Tolerance under increased salt levels has been attributed to several mechanisms that enable plant growth and development.

Kamińska et al. [88] investigated the protective mechanisms against osmotic and ionic stresses involved in the salt tolerance of carrots. In this study, the salinity EC 3.15 dS m^{-1} was maintained for the doubled-haploid DH1 line (sensitive to salinity) and DLBA (exhibiting tolerance to salinity), a local variety (Fars region in Iran), to determine the changes in biochemical traits. It was observed that the tolerant DLBA variety was moderately determined constitutively. Even the exposure to saline soil caused a physiological response, more evident in the root. Thus, carrot plants adapted to stress conditions by osmotic adjustments and activation of the antioxidant system.

It was evident that osmoprotective proline and low molecular antioxidants, such as glutathione and ascorbic acid contents, were increased, with a decreased ratio of reduced to oxidized glutathione forms. All in all, these alterations indicate an effective ascorbate–glutathione cycle operation with a high activity of antioxidative enzymes, such as peroxidases, involved in resistance against extreme reactive oxygen species.

Kwolek et al. [91] studied carrot F2 lines segregated in salt tolerance levels, derived from cross-fertilization of two lines, one resistant (DLB-A, an Iranian line) and the other susceptible (2874B, a Polish breeding line) to salinity. At 150 mM NaCl, the early response of seedlings in the germination assay indicated that salinity stress increased the time required for germination up to 4 weeks from 1 to 2 weeks, and only 20% of seedlings were grown. All salt-stressed seedlings exhibited relatively normal morphology, besides the thickening of hypocotyls, roots, and cotyledons, with a chlorotic green-yellow coloration of all organs observed. Moreover, the increased water uptake could be a vital factor in carrot tolerance to salinity. Using genotyping-by-sequencing (GBS), Kwolek et al. [91] identified the regions in the genome of the carrots that were involved in tolerance to salinity, which accounted for the lethality of F2 plants sensitive to salinity. It was based on the expected deviation from the Mendelian segregation in the group of plants under stress, whereas no deviations were expected in controls. It was revealed that the incidence of SNP alleles in the F2 plants under stress differed compared to the control plants. Most polymorphisms exhibited partial segregation on chromosome 2 in the salt-treated lines but not in the control. Moreover, in the salt-treated F2 sub-population, only one variant of chromosome 2, heredity from the tolerant parent, was conserved. It likely bears dominant gene(s) acclimatizing resistance to salinity stress.

An experiment on carrots by Simpson et al. [92] revealed salt stress and ABA (abscisic acid)-induced expression of DcPSY2 (phytoene synthase (PSY) promoter fragment) by binding of AREB transcription factors (probably DcAREB3) to the ABREs noticed in the promoter of DcPSY2. In the transcriptome of the carrot, three ABRE-binding protein (DcAREB) transcription factor candidates, localized in the nucleus, were identified. However, only one of the three, namely, DcAREB3, was induced under ABA treatment in carrot roots. AREB transcription factors were discovered in the carrot DcPSY2 promoter and expressed reporter genes by transactivation. Furthermore, the increase in the expression of DcPSY2 gave rise to the production of carotenoids. It resulted in an increase in ABA levels of resistance in the plant.

4.2. High Temperature

Nascimento et al. [93] demonstrated that high temperature inhibits carrot seed germination for some carrot germplasms but not all. In the last decades, cultivar development for sub-tropical and tropical climates has advanced rapidly with the development of cultivars such as Brasilia [94,95]. This issue is becoming extremely important in several cultivation areas due to climate change. For example, the Central Valley of California, where temperate cultivars are grown, has a typical average daytime temperature of 30 °C, with a day/night range of 24–37 °C [96]. Furthermore, if adequate water is available, carrots grow widely in warm climates, such as Tunisia, Spain, and Uzbekistan. Given a scenario for even a +4 °C increase in global temperatures (GISGeography, https://gisgeography.com/climate-c, accessed on 11 December 2018), a relatively minimal threat to carrot production might be expected for most global areas if adequate water is available, based upon the success of carrot production in the Central Valley of California today.

4.3. Drought Stress

The effects of drought stress on carrots have been scarcely documented in scientific literature. However, with the expected reduced water availability, production would likely be severely limited in most global regions without irrigation [97], although the drought response has been reported to vary widely across diverse cultivars [98]. Reduced water availability for agriculture is expected to be especially acute in Central Asia, the Middle East, North and South Africa, and the western US [99].

5. Post-Harvest Physiology

5.1. Carrots as a Perishable Food

Carrot is one of the 10 most produced crops worldwide. In 2022, the global production of carrots and turnips was over 42 million metric tons (FAOSTAT, 2024), with China being the main producer and Europe accounting for 18.8% of world production. In the last years, fresh-cut vegetables have shown an increasing trend, which has been particularly evident during and after the recent COVID-19 pandemic [100,101]. The reasons for this positive trend are the healthy image of the product, its ease of use, and the longer shelf life compared to unprocessed vegetables; however, there are also increasing concerns for the environmental impact of fresh-cut production, as well as for the human health risks deriving from the exposure to disinfection by-products that can be found in these vegetables [102]. In any case, processed carrot perishable products, namely, baby carrots, fresh-cut carrots, and vacuum-cooked carrots, are expanding their market volumes in Western countries, also as an effect of marketing initiatives, such as Eat 'Em Like Junk Food in the US in 2010 [103].

On the marketing side, the color of fresh-cut carrots is the primary sensory factor for consumers' acceptance. In fresh-cut carrots, the main color degradation processes that occur are whitening and browning. Enzymatic oxidation of polyphenolic compounds relates to the browning of carrots [104], and in UV-C-treated products, its occurrence is due to the higher peroxidase (POD) activity [105]. The whitening mechanism relates to the first rescindable physical phase of dehydration and, lately, to an irretrievable physiological response linked to activation of phenolic metabolism and production of lignin [106]. Whitening index (WI) changes do not seem to affect the visual quality of carrots [107].

The preliminary processing steps of carrots, from acceptance of raw materials to the first foreign body control, are common. Then, in uncooked chilled carrots, antimicrobial treatments are an option that many producers consider to ensure product safety and extend the shelf life (Figure 8).

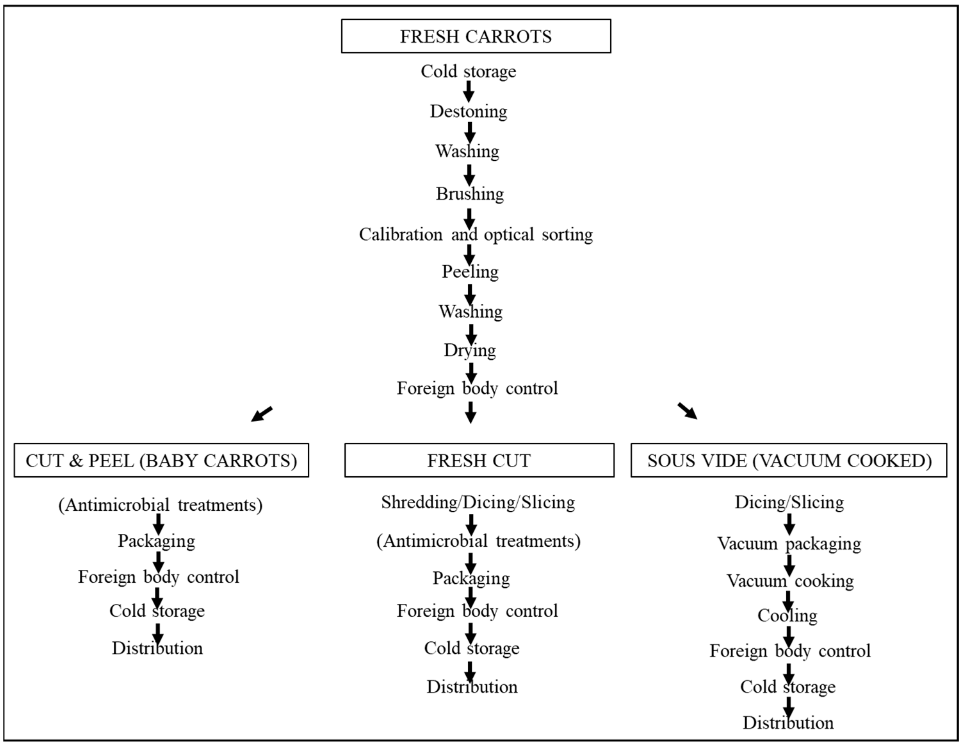


Figure 8. Representative patterns of perishable carrot products.

The feasibility of this option depends on regulatory constraints, producer policies, as well as on the agreements between manufacturers and distributors. In the European Union, if these antimicrobial treatments meet the requirements indicated in Regulation 1333/2008/EC Art. 3, they are considered technological aids and may be omitted on the label. On the other hand, antimicrobial treatments are not needed in vacuum-cooked carrots (or sous vide carrots), which are cooked in their own packaging and then cooled and stored under refrigeration.

As for the packaging used in perishable carrot products, vacuum is the only possible option for vacuum-cooked carrots, while the modified atmosphere is usually applied to baby carrots and fresh-cut carrots. All these products are commonly available in supermarkets, but sometimes they can also be found in small food stalls, where the important requirement of chilled storage can be critical.

5.2. Shelf-Life Extension

The main aspects of quality loss during post-harvest storage must be counteracted to extend the shelf life. First, lowering the respiration rate results in a longer shelf life; thus, refrigeration temperatures, modified atmospheres, and carrot coating have been the main strategies applied. Other methods, such as gaseous chlorine, ozone, and other physical technologies, have also been exploited in the later decades. These methods are mainly applied to minimally processed carrots, as washing, cutting, and slicing can stress the vegetable, increasing the exposure to spoiling microorganisms (Table 1).

Gas modification inside packaging and refrigeration can be applied to extend carrots' shelf life. Modified atmosphere packaging (MAP) generally relies on low oxygen and high carbon dioxide percentages. The effect of different storage atmospheres was studied on chopped carrots previously sanitized in 200 mg/L of free chlorine and stored at 1 °C. Vitamin C and the approximate composition did not change in the air, under vacuum, or in MAP (2% O_2, 10% CO_2, and 88% N_2), while β-carotene content decreased during storage, particularly in MAP. Microbial counts were low until the end of storage, independently of the atmosphere used, with psychotropic bacteria reaching the maximum value of 1.5×10^3 Log CFU/g after 21 days of storage in the air [108]. Similarly, a gas atmosphere composed of 5% O_2, 10% CO_2, and 85% N_2 inhibited yeast and mold growth during 21 days of storage at 4 °C, although it could not completely inhibit the development of mesophilic aerobic bacteria [109].

As already mentioned, one of the main defects of peeled carrots is the white discoloration caused by surface dehydration and lignification. To counteract this phenomenon, hygroscopic coatings made of salt solutions and polyhydric alcohols have been explored, with good results. In fact, sorbitol, glycerol, calcium chloride, calcium lactate, and propylene glycol were useful in maintaining moisture on the vegetable surface by means of a transparent layer [110]. Also, coatings based on casein, cellulose, or chitosan are effective in creating a semi-permeable barrier to oxygen and carbon dioxide, preventing moisture loss and having a preservative effect that is similar to a modified atmosphere [111]. The most suitable biopolymer is chosen based on vegetable physiology. It has the effect of slowing down the respiration rate, dehydration, gas exchange, and oxidative events, generally reducing the growth of microbial targets, thus extending the shelf life by several days and preserving qualitative and sensory attributes. Moreover, edible coatings can also be useful as carriers of anti-browning agents or antimicrobials, helping in shelf-life extension and vegetable safety improvement.

Table 1. Effects of treatments on carrots' shelf-life extension and on the product microbiota and characteristics.

Treatment	Effect on Microorganisms	Effects on Carrots' Chemical and Physical Parameters	Reference
Modified atmosphere packaging (MAP)	Growth control of the psychotropic population, inhibition of yeast and molds	Vitamin C preservation, a slight reduction in β-carotene, and minerals' content decreases during storage. Negative effect on texture, preservation of color, and quality indexes.	[108,109]
Dipping/coatings based on natural polymers (alginate, casein, chitosan, etc.)	Growth control of specific spoilage organisms, *Enterobacteriaceae* and *Pseudomonadaceae*	Reduction in flavonoids and phenolic acids' accumulation, bitterness reduction, moisture loss prevention, the anti-browning effect, color retention, and differences in antioxidant potential depending on the treatment.	[111,112]
Coatings + MAP	Load reduction and growth control of yeast and molds, coliforms, and *Pseudomonas* spp.	Moisture loss prevention, respiration increase, prolonged firmness, prevention of surface whitening, color and texture retention.	[113]
Ozonation/ozonated water	Inhibition of *Escherichia coli* O157:H7, STEC *E. coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Pectobacterium carotovorum.* Fungistatic effect on *B. cinerea* and *S. sclerotiorum*	Delay of carrot thickening, maintenance of pH, dose-dependent oxidative damages: pigment disruption, color change, increased respiratory rate, dehydration, and electrolyte loss.	[114–118]
Ozone + UV-C rays	Reduction of total mesophilic population and coliforms. No effect on yeast and molds.	Not reported.	[119]
Ozone + MAP	Inhibition of microorganisms on the product surface. Reduction of total mesophilic population.	Reduction in total phenolics, enzyme activity, respiration, and ethylene rate, retention of total carotenoids and ascorbic acid, color maintenance.	[116]
Chlorine dioxide	Reduction of mesophilic and psychrotrophic population, including lactic acid bacteria. Scarce effect on yeast that determined the shelf life.	Moisture loss prevention, white discoloration prevention, slight pH reduction, and maintenance of sensory attributes.	[120]
High pressure	Inactivation of vegetative cells.	Maintenance of texture, red color, and carotenoid content, as well as dry matter reduction. Increase of free and bound phenolics, increase of antioxidant content.	[121,122]

Table 1. *Cont.*

Treatment	Effect on Microorganisms	Effects on Carrots' Chemical and Physical Parameters	Reference
UV-C treatment	Variable inhibition of microbial growth, depending on the wavelength. Reduction in *Sclerotinia sclerotorium* load.	Maintenance of aroma, color, nutritional, and physical–chemical characteristics.	[123,124]
Gamma irradiation	Limited effect because of the legal restrictions in the doses applicable.	Maintenance of quality attributes.	[125]
Irradiation + active coating	Reduction of total mesophilic population and yeast and mold count.	Improvement of mechanical and water vapor barrier characteristics of the coating, maintenance of weight, firmness, and color.	[125]
Nisin + plant extracts + irradiation	Reduction of total mesophilic population, yeast and molds, and *Listeria monocytogenes* count.	Maintenance of weight, firmness, and color.	[126]
Different essential oils	Reduction of *Sclerotinia sclerotiorum* growth.	Increase in enzymes (polyphenol oxidase, peroxidases, chitinases, etc.) content, inducers of resistance against the molds.	[127]
Coriandrum sativum EO	Reduction of *Salmonella enterica* growth.	Maintenance of sensory traits of the product, as well as color stability.	[76]
Thyme EO	Reduction of *Escherichia coli* O157:H7 count.	Not reported.	[115]
Thyme EO + ClO_2 + ozonated water	Effective reduction of *Escherichia coli* O157:H7 count.	Not reported.	[115]
Microencapsulated chitosan + thyme EO	Reduction and control of mesophilic, psychrophilic, yeast, and mold populations during time.	Increase of total phenolics content (TPC) and antioxidant capacity.	[128]

In addition, dipping (i.e., in ethanol) and application of edible coatings (i.e., alginate) can control the product dehydration and the microbial development, particularly of specific spoilage bacteria, such as *Enterobacteriaceae* and *Pseudomonas* spp., still preserving sensory properties, with a significant shelf-life extension up to 12–13 days [112]. Furthermore, edible coatings have the advantage of being produced from fruit and vegetable by-products and residues, consequently improving the quality of the treated vegetables and converting waste into a functional film with added value. Moreover, functional coatings can be suitable for spraying and dipping, depending on the needs of the final product. In general, a larger vegetable surface exposed to the coating determines a more efficient protective effect of the layer, as the tissue absorbs part of the coating. Therefore, shredded carrots yield better results than sliced ones [129]. Chitosan powder has been directly applied to carrot shreds at concentrations up to 0.4% and stored in LDPE bags at 10 °C for 10 days. The treated samples had mesophilic loads lower than 1.3 log CFU/g with respect to the control, and the treatment also significantly reduced yeasts and molds, determining minimal pH, titrable acidity, and total solid content variations. The microbiological and sensory quality of treated samples was superior after 10 days of storage, while controls were acceptable only up to 5 days of storage [130].

Different preservation methods can also be combined, such as chitosan-based coatings, MAP, and refrigerated storage of baby carrots, with a better effect on microbial spoilage delay with respect to individually applied strategies. Chitosan alone already exerts antimicrobial activity, and the combination of all the hurdles can help control the growth of total viable microorganisms, yeasts and molds, coliforms, and *Pseudomonas* spp. over time [113].

Recently, different physical methods have been proposed for carrot treatment to extend the products' shelf life and/or improve their safety; for example, ozone, to be applied as a gas or as ozonated water [114]. Different effectiveness has been reported, depending on the duration of the treatment, ozone concentration, and the microbial target. For example, increasing the storage temperature would require more ozone to guarantee a specific residual concentration. There appears to exist a threshold in ozone concentration (up to 5 mg/L of gaseous ozone and up to 10 mg/L of ozone dissolved in water), above which the exposure can cause damage to the vegetable. Below these doses, ozone delays carrots' thickening and inhibits microorganisms, extending carrots' shelf life, although ozone in water can temporarily affect the internal pH [114]. The antimicrobial effect on *E. coli* O157:H7, STEC *E. coli*, *Salmonella enterica*, and *Listeria monocytogenes* has been proven and seems to increase with concentrations and time of exposure [115,116]. According to Hassenberg et al. [117], ozonized water at a concentration of 4 ppm for 2 min inhibited *Pectobacterium carotovorum* in washed carrots, without leaving any hazardous residue. Nevertheless, according to other authors, the effect on fungi, such as *B. cinerea* and *S. sclerotiorum*, is only fungistatic and not fungicidal. At the same time, concentrations comprised between 10 and 22 μL/L at 2 °C, because of their oxidative effect, caused physiological damages to the vegetable, including color change due to pigment destruction, and increased the respiration rate and loss of electrolytes [118]. Due to the oxidation power of ozone, terpenes can also increase in the headspace.

Chlorine dioxide (ClO_2) has been studied for the shelf-life extension of minimally processed carrots. Unlike liquid chlorine and hypochlorite, chlorine dioxide does not react with ammonia-forming chloramines, which are toxic for workers and consumers, but still behaves as a strong oxidizing agent with an antimicrobial effect on surfaces. This gas can inhibit microbial growth, keep carrot tissues hydrated, and thus reduce the risk of white blush discoloration [120]. A treatment of 6 min at 28 °C with a maximum ClO_2 concentration of 1.33 mg/L significantly reduced mesophilic and psychotropic bacteria, particularly lactic acid bacteria, preserving the sensory attributes of carrot sticks. Unfortunately, the treatment was less effective on yeasts, which allowed a shelf-life extension of only one day, reaching a load greater than 5 Log CFU/g after five days of storage [120].

During the last decades, high-pressure processing (HPP) has emerged as a non-thermal process in which food products are subjected to a pressure of 400–600 MPa at room or refrigerated temperature for a variable time of a few minutes [121]. The treatment inactivates vegetative microbial cells, extending the products' shelf life. Specifically, in carrots, HPP treatments guarantee better texture preservation and red color retention than thermal treatments, considering treatments with an equivalent effect on microbial inactivation [121]. Moreover, HPP processing of whole carrots at mild conditions (60–100 MPa for 5 min) can increase the content of natural antioxidants, such as free and bound phenolic compounds, preserving the carotenoid content [122].

Among the physical methods, UV-C treatment is a non-thermal disinfection method mainly used for surfaces. The strongest antimicrobial effect is reached when radiation at 253.7 nm is applied. The radiation hits the microbial DNA, preventing its transcription and translation and, therefore, inhibiting microbial growth. The treatment generally maintains the qualitative, nutritional, and physical–chemical characteristics of the product, without affecting aroma and color [123]. The application of UV-C radiation with a peak at 254 nm for 5 min significantly decreased the *S. sclerotorium* load [124]. Nevertheless, some authors applied UV-C at 253 nm on carrots and obtained a microbial reduction of about 1 Log CFU/mL, which was insufficient for shelf-life extension [123].

Gamma irradiation is another physical, non-thermal method to assure food safety, which can be applied to fresh vegetable products. Although considered safe for consumers by the Codex Alimentarius Commission, World Health Organization, FAO, and International Atomic Energy Agency, food irradiation is not allowed in all nations. Still, it has been approved in over 60 countries [131]. Nonetheless, the doses necessary to inhibit pathogenic microorganisms to an undetectable level usually exceed the dose recommended for fruit and vegetables, which is below 1 kGy, although the nutritional quality of this product is preserved at irradiation doses up to 10 kGy [125]. Consequently, the approach based on the "hurdle technology" is frequently applied to overcome the limited antimicrobial effectiveness of these physical methods. In detail, combining different preservation methods or techniques can improve food safety and extend the shelf life without reducing the nutritional and sensory quality of the product. For example, the combination of ozone and UV-C rays [119], ozone with modified atmosphere [116], or even irradiation and bioactive coating based on calcium caseinate incorporated with citrus extract, cranberry juice, and essential oils [125], and nisin plus carvacrol or mountain savory and then irradiation at 0.5 and 1.0 KGy [126], have been proposed. The combination of different hurdles and/or technologies generally shows a synergistic potential and a higher efficiency in extending carrots' shelf life, compared with the same treatments singularly applied.

Finally, essential oils and plant extracts have also been applied to counteract microbial pathogens' growth or extend the shelf life of carrots. The effects of different essential oils (EOs) were tested against *Sclerotinia sclerotiorum* during carrots' storage. Thyme and savory EOs not only were the most effective but also increased the level of peroxidases, chitinases, polyphenol oxidase, and other enzymes, therefore showing the potential to induce resistance of the vegetable against the white mold [132]. In another study, Pellegrini et al. [76] applied *Coriandrum sativum* essential oil (5 µL/mL) as a washing treatment on carrot sticks. Two minutes of contact reduced the load of a cocktail of three *Salmonella enterica* strains of about 1 Log CFU/g for up to 24 h, without affecting the sensory profile of the product. The authors suggested applying this washing treatment in combination with other treatments to boost this effect. While washing baby carrots for 5 min with thyme essential oil (1.0 mL/L) determined more than a 1.0 Log CFU/g reduction of inoculated *Escherichia coli* O157:H7, the sequential washing by means of thyme EO, ClO_2, and ozonated water was significantly more effective, reducing the *E. coli* load by more than 3.75 Log CFU/g [115]. A chitosan coating containing free thyme EO and microencapsulated in β-cyclodextrin was demonstrated to exert antimicrobial activity on mesophilic, psychrophilic, yeasts, and mold populations and to extend the shelf life of sliced carrots. While the first combination immediately reduced the microbial count, the effect was lost during storage. The coating containing microencapsulated thyme EO reduced the load from day 6 until the end of storage [128].

As a result of all the studies previously described, a combination of different techniques is the most effective strategy to extend carrots' shelf life from microbiological, nutritional, sensory, and technological points of view.

6. Carrot Breeding: Genetic Resources and Genomic Selection

Carrot accessions in the germplasm are the treasured sources of desired traits with genetic diversity. The genotyping of total accessions in germplasm collections makes it likely to use genomic prediction for valued accession identification and exclude less desired trait values [133]. Genomic prediction of accessions by different strategies offers a convincing landscape of breeding programs over field screening. The introgression of desired traits as accessions into stand-out breeding lines by means of genomic and phenotypic selection leads to new genetic makeup for improving carrot varieties.

D. carota is a cross-pollinated diploid species. It is vital in human nutrition and agroeconomy [134]. Carrots, canopy height, and flavor are quantitative traits with moderate heritability. In breeding for weeds, lowness vs. height of the canopy is a goal in the selection process [135,136]. Several carrot accessions have harsh flavors due to volatile terpenoids, but selected varieties typically have mild (non-harsh) features. Upholding mild flavor while breeding canopy height traits into elite breeding lines is now an engrossed breeding goal [43].

In fact, the engrossed breeding goal for carrots is to breed varieties with tall canopies and mild flavor as a model vegetable crop. In breeding contexts, genomic prediction strategies can assist in identifying valuable breeding material with high-density genotype data to predict phenotypes or breeding values in collections/germplasms. The application of genomic prediction requires designated populations to be estimated in a potential breeding context. The genomic selection will possibly allow the identification of valuable accessions without requiring extensive field evaluation. Therefore, it appears to yield similar results as phenotypic selection, with lower costs for phenotyping. Thus, assessing a training population in the target environment may be strategic for some traits [137].

Corak et al. [137] compared the performance of two genomic selection strategies. The first method, the genomic-breeding population (GBP), uses past data of phenotypes to forecast the accession with a genetic value of additives so that field screening of accessions in selecting parents is avoided. The second method, the genomic-training population (GTP) method, employs data from a training population of representative phenotypes in an environment of the target [138]. These methods have limits to assessing accessions in the field, with strong access to genotypic data. These two genomic selection strategies with phenotype selection (PS) were tested to identify carrot accessions with a tall canopy height and good flavor. In this study, a selection model trained on phenotypes from only 10% of the collection was found to be the most promising. This means that the trade-off in prediction accuracy and the cost of phenotyping could be balanced using an optimum training population size, which is key to identifying and excluding unwanted accessions. The results obtained in [137] demonstrated that populations derived from crosses between highly ranked accessions of parental and selective inbreds showed similar trait distributions. With additional cycles in selection, the results of the GTP group selection at the F2 generation can be considered encouraging, as reported by Corak et al. [138]. Even in a larger training population, GTP would reduce the expanse of phenotyping needed prior to selecting parental accessions for target traits.

In the future, the improvement of carrots is likely to be assisted by artificial intelligence and machine learning methods, which can analyze the correlation between various attributes, such as yield and nutritional characteristics. This issue is extremely relevant for human nutrition, considering that 28–90% of total β-carotene for humans comes from carrots. Moreover, the data on the nutritional traits of genotypes complement essential phenotypic and genetic characterization and its association with color variation. Riaz et al. [139] reported that various morpho-nutrition traits were estimated in 64 genotypes collected from 4 continents. An evaluation of genetic variability, heritability, strength, and direction of association among variables, and direct and indirect relationships among physicochemical and nutritional traits with β-carotene content was assessed. A significant association with β-carotene accumulation was noted with core diameter, foliage weight, root weight, and shoulder weight. Principal component analysis divided genotypes into two typical groups: Eastern and Western carrots. It was revealed that caloric and moisture content had high positive links with β-carotene content, while carbohydrate content was negatively associated. In this study, five genotypes (T-29, PI 634658, PI 288765, PI 164798, and Ames 25043) with the highest β-carotene contents were selected and used for making three nutraceutical supplements (carrot–orange juice, carrot jam, and carrot candies). These nutraceutical supplements retained a high β-carotene content coupled with antioxidant properties [139].

Koutouan et al. [44] screened a total of 300 accessions from carrot genetic resources in Angers (France) and other European genetic resources from 1997 to 2000 for their resistance to *Alternaria dauci* in different environments. Based on the authors' findings, three inbred lines, namely K3, I2, and Boléro, were highly resistant, whereas genotype H1 was highly susceptible. The selection was based on several quantitative trait loci (QTL) associated with resistance to *A. dauci.* The different genotypes showed varied resistance mechanisms for the QTLs involved [140,141]. The strategy described by Koutouan et al. [44] can be useful to identify the genotype with the highest resistance.

7. Future Prospects

For carrot seed companies, one of the core breeding objectives is to increase the resistance level of new cultivars in one genotype by accumulating complementary resistance factors while breeding for less weeds. Canopy height and flavor are the two quantitative traits that uphold the mild flavor. In contrast, breeding canopy height into elite breeding lines is now an engrossed breeding goal.

Although carrots are a well-known and explored staple food, increasing efforts to improve their safety and shelf life have been documented in recent literature. In this respect, also in view of the challenges generated by climate change, future strategies for post-harvest storage and processing are likely to be based on a combination of methods. Finally, in the framework of circular economy, carrots will become a source of important bioactive compounds and by-products, which will be explored in different industrial environments. In this respect, the data gathered in this review can be considered a valuable toolbox for both crop scientists and food technologists.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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A high-efficiency 3-Step in vitro protocol for commercial cormel micropropagation of Gladiolus hybridus Hort. cv 'Red Majesty'

Article *in* International Journal of Plant Research · December 2024

DOI: 10.1007/s42535-024-01134-5

3 authors, including:

Madeeha Naik
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Amjad M. Husaini
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Vegetos
https://doi.org/10.1007/s42535-024-01134-5

RESEARCH ARTICLES

A high-efficiency 3-Step in vitro protocol for commercial cormel micropropagation of *Gladiolus hybridus* Hort. cv 'Red Majesty'

Madeeha Naik[1] · **Imtiyaz T. Nazki**[2] · **Amjad M. Husaini**[1]

Received: 20 December 2023 / Revised: 26 November 2024 / Accepted: 4 December 2024

Abstract

The present study aimed to standardize a cormel in vitro propagation protocol for *Gladiolus hybridus* Hort. cv 'Red Majesty'. The study evaluated many parameters, from culture establishment to cormlet development of *Gladiolus hybridus* Hort. There was a significant effect of treatments on these parameters and a 3-step process was standardized for its commercial micropropagation. Minimum (45) days to cormlet appearance was recorded on MS + B_5 vitamins supplemented with CCC ($1 mgl^{-1}$) + sucrose (9%), while the maximum average number of cormels per explant (14) was produced on MS + B_5 vitamins supplemented with IBA ($0.5\ mgl^{-1}$) + CCC ($2\ mgl^{-1}$) + sucrose (3%). Maximum cormel development percent (85) was observed on MS + B5 vitamins supplemented with growth regulator combination CCC ($1.0 mgl^{-1}$) + sucrose (9%). Maximum number of cormels (14) was observed in (IBA $0.5 mgl^{-1}$ + CCC $2 mgl^{-1}$ sucrose 3%). Overall the best treatment for the combined effect on cormlet development (w.r.t. cormlet maturation percentage and the number of cormels) was MS + B_5 medium with IBA ($0.5\ mg^{-1}$) + CCC ($2\ mg^{-1}$) + sucrose (6%) as 12 number of cormels per explant and 85 cormlet development percent was achieved. In 150 days, 12–14 cormels were obtained from a single corm slice, and each cormel size was approximately 22 mm. Therefore, the multiplication rate of superior gladiolus hybrid 'Red Majesty' can be increased 14-fold while reducing the multiplication duration using the in vitro protocol developed in the present study.

Keywords Gladiolus · Micropropagation · Tissue culture · Cormel · Cormlet

Introduction

Gladiolus is a genus of perennial herbaceous flowering plants belonging to the family Iridaceae. The genus is large, with about 260 species, of which 250 are native to sub-Saharan Africa mostly South Africa (He et al. 2008). Gladiolus is a monocot whose species are often called "Sword Lily" due to its sword-shaped leaves. The plants grow from corms that are enveloped in several layers of brownish tunics. The flowers come in numerous colours, such as white, pink, red, purple, yellow, orange, salmon, green and even several bi-colour flowers are also found. Due to the very low natural propagation rate, it takes many years to release new cultivars of gladiolus. The "Red Majesty" cultivar is a giant exotic hybrid cultivar (Blackjack × Friendship). It bears 16 florets per spike in two rows, an ideal commercial cut flower characteristic. Moreover, being red-coloured, the variety is amenable to yield novel colour mutants through in vitro mutagenesis. Plant height is 131.0 cm, spike length 95 cm, rachis length 77 cm, and the number of florets per spike is greater than 16.6 (DFR-Annula-Report-2018–19.pdf (icar.gov.in).

Gladiolus is propagated primarily by natural corm and cormel multiplication (Hartmann et al. 2002), (Ziv and Lilien-Kipnis 1990). However, these methods have many limitations (Kumar et al. 2024). One is the low rate of its multiplication in its commercial cultivation. Second is the corm rot caused by Fusarium, and high percentages of corm spoilage during storage which limits commercial corm/cormel supply. Third, four seasons are required to attain a standard flowering spike and adequately sized daughter corm. Fourth is the dormancy of corms, which causes scarcity of planting material. Fifth is the infection of viruses, which makes

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✉ Amjad M. Husaini
amjadhusaini@skuastkashmir.ac.in

[1] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, SKUAST-K, Shalimar, Srinagar, Jammu and Kashmir, India

[2] Division of Floriculture and Landscape Designing, Faculty of Horticulture, SKUAST-K, Shalimar, Srinagar, Jammu and Kashmir, India

vegetative propagation slow in gladiolus. Consequently, traditional propagation methods are inadequate to satisfy the demand for planting materials for commercial cultivation. With these bottlenecks to its mass propagation using conventional techniques, plant tissue culture is the best available alternative for its commercial production.

Plant tissue culture has been used for micropropagation of gladiolus for a long time and has been recently reviewed for progress in the last 50 years (Kumar et al. 2024). Using various hormones such as 2,4-D, IAA, BAP, and minor modifications in protocols, several studies have reported the successful micropropagation of different gladiolus cultivars (Belanekar et al. 2010; Emek 2007; Misra and Singh 1999; Nhut et al. 2004; Pathania et al. 2001; Priyakumari and Sheela 2006; Rakosy-Tican et al. 2012; Shaheenuzzaman et al. 2011). These reports generally underscore differential cultivar responses because of their genetic differences, and no micropropagation studies have been reported on the cultivar 'Red Majesty'. As the response of plants to plant tissue culture is genotype-dependent, so there is a need to develop an efficient protocol for this highly valuable cultivar. Moreover, the cost of producing in-vitro propagated plants needs to be decreased, and the return on investment increased (Savangikar 2004; Jo et al. 2008).

The present study aimed to develop an efficient micropropagation protocol for the 'Red Majesty' cultivar, which could be used for the commercial production of low-cost cormels, and evaluate commonly used growth regulators to cut costs and study their effect on bud sprouting, shoot multiplication and cormlet development of gladiolus.

Materials and methods

Materials

Standard tissue culture grade chemicals for preparation of MS (Murashige and Skoog 1962) medium, B5 vitamins, sucrose, agar and plant growth regulators were procured from Hi-Media Labs, India. The healthy cormels of gladiolus cv. "Red Majesty" were obtained from the Division of Floriculture and Landscape Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar. The stock plants were grown in small pots containing a sterilized mixture of sand: soil (1:1 ratio). They were incubated inside a walk-in Plant growth chamber at 25 ± 2 ℃ under 16/8 h photoperiod conditions.

Explant preparation

The corm sections were cleaned in running tap water, and the outer tunics were removed. Corm sections containing at least one bud were washed with tween-20, then treated with Carbendazim (50% WP) for 30 min and washed thrice. The surfactant and fungicide were washed off with single distilled water. After the initial cleaning, the explants were brought to a laminar flow hood for treatment with 0.1% mercuric chloride for 10 min, then washed three times with sterilized distilled water (Hussain et al. 2001). Corm sections were placed on the medium for aseptic inoculation, conforming to their original polarity.

Culturing

Corm sections were placed on the medium for bud sprouting and shoot multiplication in 25 mm × 150 mm conical flask containing Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of growth regulators (BAP, KIN and GA_3) (Tables 1 and 2). For the development of cormels, the shoots were placed on the MS medium supplemented with different concentrations and combinations of growth regulators and sucrose and growth inhibitors like 2-Chloro-N,N,N-trimethylethan-1-aminium (also called Chlorocholine chloride; Chlormequat chloride; Cycocel; CCC), which is used as a foliar spray to decrease stolon growth and increase tuber yield in potato (Wang et al. 2009) (Tables 3, 4 and 5). The culture flasks were incubated inside the culture room at 25 ± 2 ℃ under 8/16 h photoperiod conditions.

Statistical analysis

The experiment was set up in a Completely Randomized Design (CRD), comprising four replicates for each treatment. Statistical analysis was done for all results using Analysis of Variance (ANOVA) in OPSTAT (Sheoran et al. 1998), and the treatment effects were plotted as bar graphs using GraphPad Prism 8.0.2.263.

Results

Bud sprouting

In the present study, bud sprouting of *Gladiolus hybridus* Hort. cv "Red Majesty" was gauged in terms number of sprouts, days to sprouting and sprouting percentage (Table 1) (Fig. 1 a,b,c). Sprouting percentage varied significantly under different growth regulator combinations (Table 1). It is clear that BAP $0.5 mgl^{-1}$ in combination with GA_3 $2 mgl^{-1}$ increased the sprouting percentage, but at higher concentrations of BAP sprouting percentage decreased. Higher sprouting percentage (91.25%) was recorded on growth regulator combination BAP ($0.5 mgl^{-1}$) + GA_3 ($2.0 mgl^{-1}$) followed by 82.50% on BAP

Table 1 Effect of plant growth regulators on culture establishment in *Gladiolus hybridus* cv "Red Majesty"

Treatments (MS + PGRS)	Days to sprouting	Number of sprouts /explant	Sprouting %age
T1: BAP (0.5 mgl^{-1})	5.40 ± 0.2	1.25 ± 0.2	48.50 (44.1)
T2: BAP (1.0 mgl^{-1})	6.55 ± 0.3	2.00 ± 0.4	42.75 (40.8)
T3: BAP (1.5 mgl^{-1})	8.12 ± 0.3	3.00 ± 0.4	38.25 (38.1)
T4: BAP (2.0 mgl^{-1})	9.95 ± 0.4	**4.00 ± 0.0**	30.50 (33.5)
T5: BAP (0.5 mgl^{-1}) + GA$_3$ (1.0mgl^{-1})	4.17 ± 0.2	1.15 ± 0.1	78.25 (62.1)
T6: BAP (1.0 mgl^{-1}) + GA$_3$ (1.0mgl^{-1})	5.20 ± 0.4	2.00 ± 0.0	65.75 (54.1)
T7: BAP (1.5 mgl^{-1}) + GA$_3$ (1.0mgl^{-1})	6.42 ± 0.5	2.80 ± 0.1	50.75 (45.4)
T8: BAP (2.0 mgl^{-1}) + GA$_3$ (1.0mgl^{-1})	7.50 ± 0.3	3.82 ± 0.1	41.25 (39.9)
T9: BAP (0.5 mgl^{-1}) + GA$_3$ (2.0mgl^{-1})	**2.75 ± 0.4**	1.00 ± 0.0	**91.25 (72.9**)
T10: BAP (1.0 mgl^{-1}) + GA$_3$ (2.0mgl^{-1})	3.87 ± 0.1	1.97 ± 0.0	82.50 (65.2)
T11: BAP (1.5 mgl^{-1}) + GA$_3$ (2.0mgl^{-1})	5.17 ± 0.3	2.70 ± 0.2	72.75 (58.1)
T12: BAP 2.0 mgl^{-1}) + GA$_3$ (2.0mgl^{-1})	6.20 ± 0.3	3.50 ± 0.2	61.00 (51.3)
C.D ($P \leq 0.05$)	1.05	0.66	3.35 (2.30)

Figures in the parentheses are arc sign transformed values of percentage data

Data recorded after 4 weeks of culture

Table 2 Effect of plant growth substances on multiple shoot proliferation of *Gladiolus hybridus* cv "Red Majesty"

Treatments (MS + PGRS)	Days to shoot appearance	Number of shoots/explant	Shoot length (cm)	Leaf number per shoot
T1: BAP (1.0mgl^{-1} + Kinetin (0.5mgl^{-1})	7.22 ± 0.1	4.5 ± 0.2	9.25 ± 0.6	1.0 ± 0.0
T2: BAP (2.0mgl^{-1}) + Kinetin (0.5mgl^{-1})	6.35 ± 0.2	6.5 ± 0.2	7.25 ± 0.2	1.2 ± 0.25
T3: BAP (3.0mgl^{-1}) + Kinetin (0.5mgl^{-1})	5.22 ± 0.1	9.0 ± 0.4	5.00 ± 0.4	2.5 ± 0.8
T4: BAP (1.0 mg^{-1}) + Kinetin (1.0mgl^{-1})	7.00 ± 0.0	11.0 ± 0.5	4.00 ± 0.8	2.7 ± 0.8
T5: BAP (2mgl^{-1}) + Kinetin (1.0mgl^{-1})	6.00 ± 0.0	13.0 ± 0.7	2.5 ± 0.2	3.5 ± 0.2
T6: BAP (3.0mgl^{-1}) + Kinetin (1.0mgl^{-1})	5.00 ± 0.0	**15.0 ± 1.0**	2.00 ± 0.4	5.2 ± 0.6
T7: BAP (1.0mgl^{--1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (0.25mgl^{-1})	4.27 ± 0.1	7.75 ± 0.4	12.00 ± 0.7	6.0 ± 0.4
T8: BAP (2.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (0.5 mg^{-1})	3.32 ± 0.1	5.75 ± 0.4	14.5 ± 0.6	7.2 ± 0.4
T9: BAP (3.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (1.0mgl^{-1})	**2.00 ± 0.0**	3.2 ± 0.2	**17.00 ± 0.9**	**9.0 ± 0.4**
C D	0.36	1.64	1.76	1.56

*Data recorded after 4 weeks of sub culture

(1.0mgl^{-1}) + GA$_3$ (2.0mgl^{-1}). The lowest sprouting percentage was recorded (30%) on BAP (2.0mgl^{-1}).

The maximum number (4.0) of sprouts/explant was recorded on growth regulator combination BAP (2.0mgl^{-1}) followed by three on BAP (1.5mgl^{-1}). The minimum number of sprouts (1.0) was recorded on BAP (0.5 mg l^{-1}) + GA$_3$ (2.0 mg l^{-1}). Minimum (2) days to sprouting was recorded on growth regulator combination BAP (0.5mgl^{-1}) + GA$_3$ (2.0mgl^{-1}) followed b by (3.8) on BAP (1mgl^{-1}) + GA$_3$ (2mgl^{-1}). Maximum (9.95) days to sprouting was recorded on BAP (2.0mgl^{-1}) followed by (8.12) on BAP (1.5mgl^{-1}).

Shoot proliferation

In the present investigation, shoot proliferation was studied in terms of days to shoot appearance, shoot number explant^{-1}, shoot length and leaf number per shoot (Fig. 1 d,e,f,g). Data about days to shoot emergence, shoot length (cm), number of shoots per explant, and leaf number per shoot were recorded after 4 weeks of subculture (Table 2). The perusal of data revealed that cytokinins play a vital role in shoot proliferation. Moreover, minimum days to shoot emergence (2) was recorded on BAP (3mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (1.0mgl^{-1}) followed

Table 3 Effect of growth regulators, sucrose and CCC on corm production of *Gladiolus hybridus* cv "Red Majesty"

Treatments (MS + PGRS)	Days to cormlet appearance	
	From sprouting of bud	After shoot multiplication
T1: CCC (1.0mgl^{-1}) + sucrose (3%)	130 ± 3.7	55 ± 2.3
T2: CCC (1.0mgl^{-1}) + sucrose (6%)	124 ± 1.6	49 ± 1.7
T3: CCC (1.0mgl^{-1}) + sucrose (9%)	**120 ± 1.4**	**45 ± 1.9**
T4: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (3%)	146 ± 1.3	71 ± 1.1
T5: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (6%)	137 ± 1.5	62 ± 1.4
T6: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (9%)	133 ± 1.4	58 ± 1.0
T7: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (3%)	143 ± 1.1	68 ± 0.8
T8: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (6%)	134 ± 2.1	59 ± 1.5
T9: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (9%)	127 ± 2.5	52 ± 2.0
CD	5.9	4.7

Data was recorded after 9 weeks of subculture

Table 4 Effect of plant growth regulators, sucrose and CCC on corm production and shoot multiplication of *Gladiolus hybridus* cv "Red Majesty"

Treatments (MS + PGRS)	Number of shoots/Explant	Shoot length (cm)	Leaf number per shoot	Number of corms per explants	Cormlet development percent	Corm size
T1: CCC (1.0mgl^{-1}) + sucrose (3%)	6.00 ± 0.0	6.50 ± 0.2	2.50 ± 0.2	2.0 ± 0.7	74.5 (59.7)	7.0 ± 0.9
T2: CCC (1.0mgl^{-1}) + sucrose (6%)	7.50 ± 0.4	8.25 ± 0.4	3.97 ± 0.0	4.0 ± 0.8	80.5 (63.8)	9.25 ± 1.8
T3: CCC (1.0mgl^{-1}) + sucrose (9%)	8.00 ± 0.0	10.75 ± 0.2	4.97 ± 0.0	6.75 ± 0.6	**85.2 (67.5)**	**11 ± 1.0**
T4: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (3%)	**20.5 ± 0.2**	**26.5 ± 0.8**	**12.0 ± 0.0**	10.2 ± 0.6	54.2 (47.4)	1.25 ± 0.2
T5: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (6%)	18.5 ± 0.6	24.0 ± 0.8	10.75 ± 0.2	8.5 ± 0.6	60.5 (51)	4.0 ± 0.7
T6: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (9%)	16.0 ± 0.4	21.7 ± 0.6	9.67 ± 0.1	3.0 ± 0.7	66.2 (54.4)	5.0 ± 0.8
T7: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (3%)	13.0 ± 0.5	18.0 ± 1.0	8.25 ± 0.2	**14 ± 0.9**	56.2 (58.5)	3.25 ± 0.6
T8: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (6%)	9.92 ± 0	15.0 ± 0.8	7.00 ± 0.0	12.2 ± 0.6	69.5 (56.4)	6.00 ± 1
T9: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (9%)	8.72 ± 0.2	13.2 ± 1.3	6.00 ± 0.0	3.2 ± 0.6	76.5 (61)	8.00 ± 0.7
CD	1.1	2.3	0.48	2.0	4.1	2.3

Data was recorded after 9 weeks of subculture

Figures in the parentheses are arc sign transformed values of percentage data

by BAP (2.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (0.5mgl^{-1}) (Fig. 1d). The highest shoot length (17.00 cm) was recorded on BAP (3.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (1.0mgl^{-1}), while the lowest shoot length (2.0 cm) was recorded in BAP (3.0mgl^{-1}) + Kinetin (1.0mgl^{-1}) (Fig. 1f).

The maximum average number of shoots per explant (15.00) developed on growth regulator combination BAP (3.0mgl^{-1}) + Kinetin (1.0mgl^{-1}). This treatment combination was followed with BAP (2.0 mg l^{-1}) + Kinetin (1.0mgl^{-1}), recording 13 shoots explant^{-1}. However minimum number of shoots per explant (3.2) was observed on BAP (3.0 mg^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (1.0mgl^{-1}) (Fig. 1e).

A perusal of the data revealed that the highest leaf number per shoot (9) developed in cultures incubated on growth regulator combination BAP (3.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (1.0mgl^{-1}) followed by 7.2 on BAP (3.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA$_3$ (0.5mgl^{-1}). The lowest leaf number per shoot (1.0) was recorded on growth regulator combination BAP (1.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) followed by 1.2 on BAP (2.0mgl^{-1}) + Kinetin (0.5mgl^{-1}) (Fig. 1g).

Table 5 Maximization of corm size transferred from different corm development media on MS half strength + 6% sucrose

Treatments from which sub-cultured		Corm size (mm)
T1: CCC (1.0mgl^{-1}) + sucrose (3%)		14 ± 1
T2: CCC (1.0mgl^{-1}) + sucrose (6%)		16 ± 0.8
T3: CCC(1.0mgl^{-1}) + sucrose (9%)		**22 ± 0.9**
T4: IBA (0.5mgl^{-1}) + CCC(1.5mgl^{-1}) + sucrose (3%)		4.0 ± 0.8
T5: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (6%)		8.0 ± 0.4
T6: IBA (0.5mgl^{-1}) + CCC (1.5mgl^{-1}) + sucrose (9%)		9.2 ± 0.8
T7: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (3%)		6.7 ± 1.2
T8: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (6%)		10 ± 1.4
T9: IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (9%)		12 ± 0.7
CD	Subcultured to half strength MS medium	2.8

Data was recorded after 4 weeks of subculture

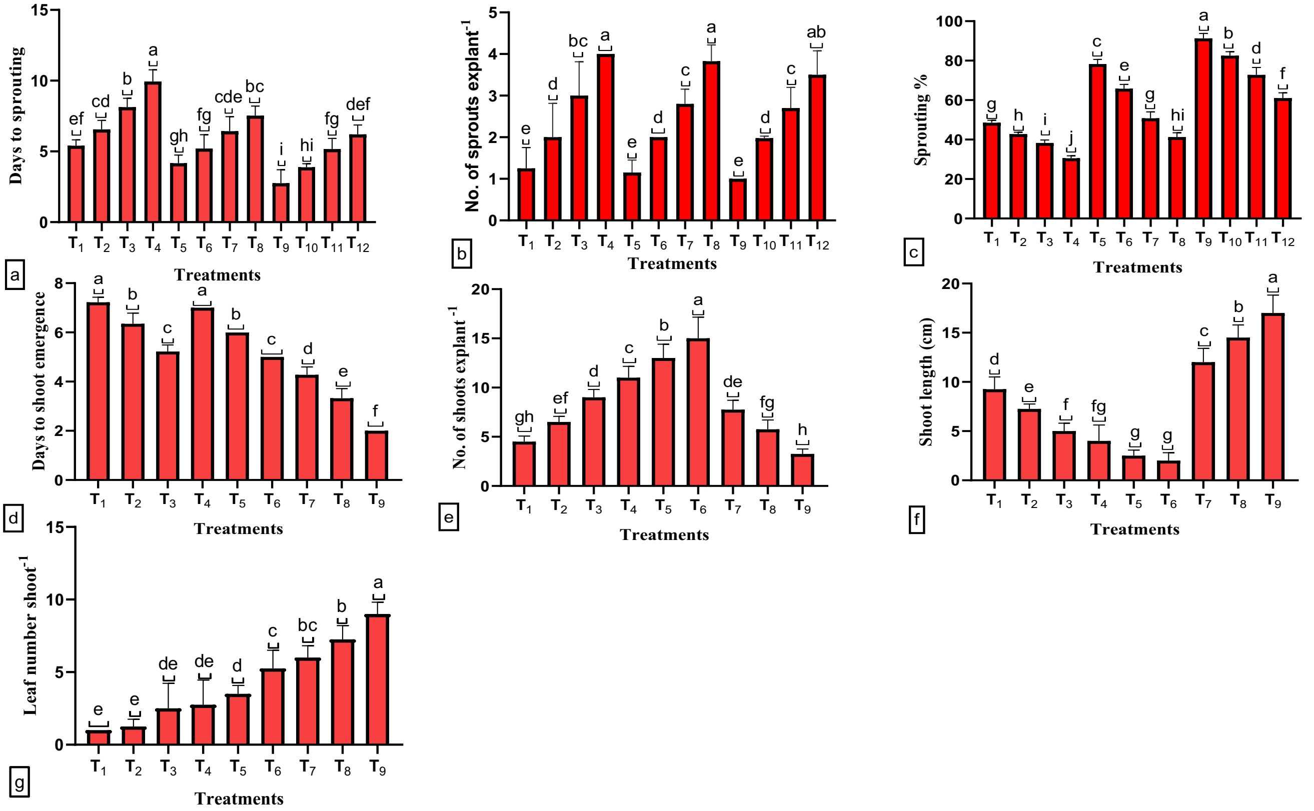

Fig. 1 Effect of plant growth substances on culture establishment and multiple shoot proliferation: **a** Days to sprouting; **b** Number of sprouts per explant; **c** Sprouting percentage; **d** Days to shoot emergence; **e** Shoot number; **f** Shoot length; **g** Leaf number

Corm development

Corm development was gauged in terms of days to corm initiation, the number of cormels, corm size, and corm development percent (Fig. 2a–h). The minimum (45) days to cormlet appearance were recorded on MS + B$_5$ vitamins supplemented with CCC (1.0mgl^{-1}) + sucrose (9%) (Fig. 2b), while the maximum number of cormels per explant (14) was produced on MS + B5 vitamins supplemented with IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (3%) (Fig. 2c). Maximum cormel development percent (85) was observed on MS + B5 vitamins supplemented with growth regulator combination CCC (1.0mgl^{-1}) + sucrose (9%) (Fig. 2d). Corm size varied with the change of sucrose concentration in the growth media. Maximum (11 mm) corm size was observed on growth regulator combination CCC (1.0mgl^{-1}) + sucrose

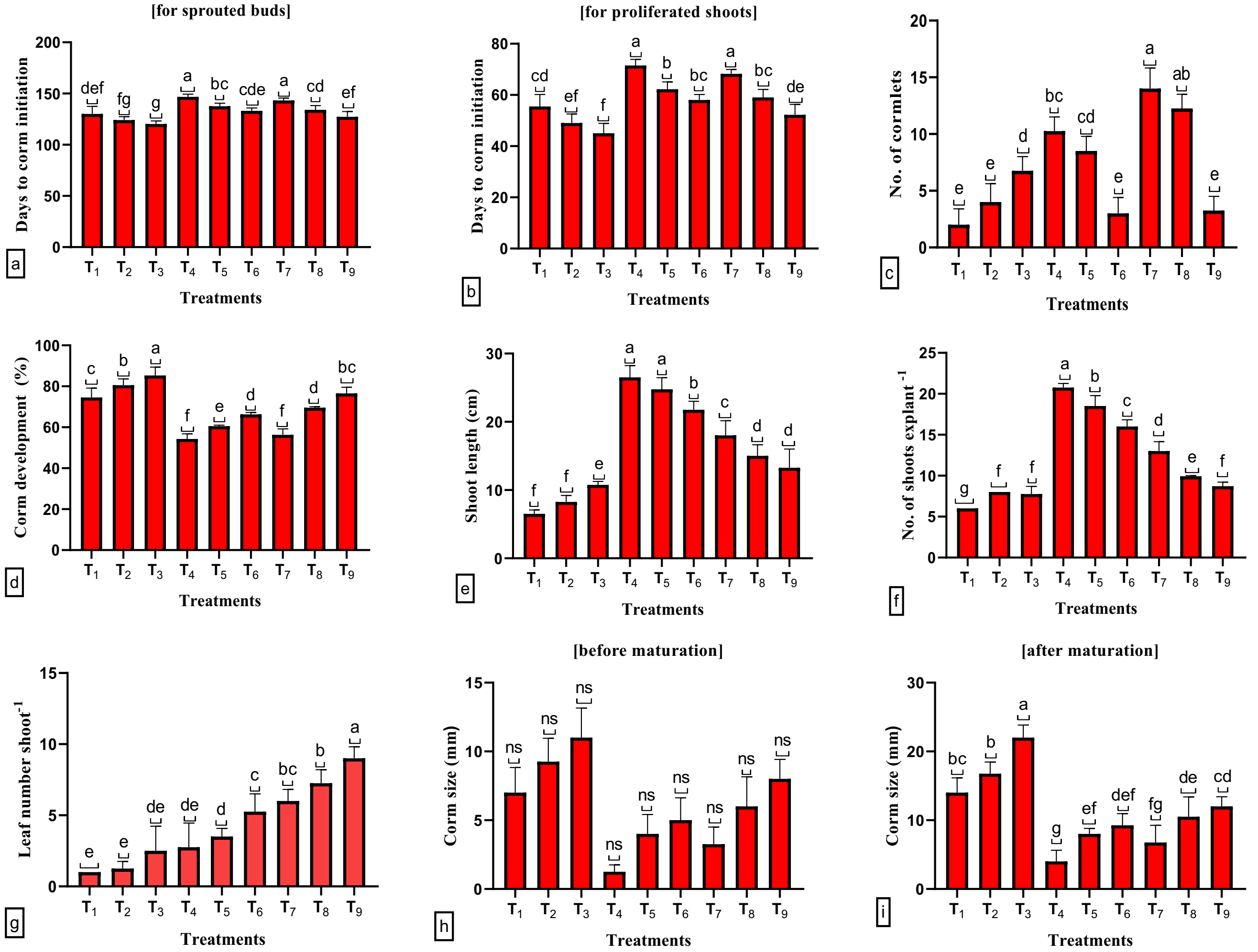

Fig. 2 Effect of plant growth substances on corm development: **a** Days to corm initiation from sprouted buds; **b** Days to corm initiation from proliferated shoots; **c** Number of cormels; **d** Corm development percentage; **e** Shoot length; **f** Shoot number; **g** Leaf number per shoot; **h** Corm size (before maturation); **i** Corm size (after maturation)

(9%) (Fig. 2g). Cormels were subcultured on half-strength MS media containing sucrose (6%) for corm maturation. After four weeks of sub-culturing, maximum corm size (20 mm) was observed in cultures that were subcultured from CCC (1.0mgl^{-1}) + sucrose (9%) on half-strength MS medium with sucrose (6%) (Fig. 2h).

Discussion

For effective micropropagation and cormel growth, the chemical composition of the culture media is critical. Murashige and Skoog medium (Murashige and Skoog 1962) along with various auxins and cytokinins, is suitable for shoot initiation, multiplication, and rooting in Gladiolus tissue culture (Lilien-Kipnis and Kochba 1985), (Logan and Zettler 1984). Axillary buds (Boonvanno and Kanchanapoom 2000), (Begum and Haddiuzaman 1995) shoot tip (Hussain et al. 2001)), cormels (Nagaraju and Parthasarathy 1995), (Wahocho et al. 2016; Wilfret 1980) and inflorescence axes are generally used to multiply gladiolus in vitro (Ziv and Lilien-Kipnis 1996). Furthermore, the cormel development is improved by adding growth retardants and increasing sucrose concentration (Ziv 1989), (Steinitz et al. 1991).

Culture asepsis

Choosing the correct explant is essential if the desired outcome of any tissue culture procedure is to be achieved with minimal delays. Besides, proper sterilization of the explants is the pre-requisite step for developing a successful protocol for in vitro propagation (Bhat et al. 2022), (Yasmin et al. 2013). Washing explants under running tap water for 30–40 min can assist in obtaining sterile cultures by physically removing some contaminants, especially with field-grown material (Jones et al. 1979). In the present study, corm-sections containing at least one bud were washed with tween-20 (detergent) and then treated with 0.3% carbendazim (50 WP) for 30 min. In a separate study, a lower concentration of 0.1% carbendazim (50 WP) was used, but the duration of treatment was prolonged to 2 h (Singh et al. 2012). The buds of gladiolus require treatment for a lesser duration (4–5 min) with 0.1% carbendazim (50 WP) in combination with 70% ethanol for sterilization (Kumar et al. 2018). Corm sections were placed on the medium for aseptic inoculation, conforming to their original polarity.

Bud sprouting

The number of sprouts per explant was the highest at a higher BAP concentration (2.0mgl^{-1}) (Fig. 3a). In comparison, at a lower concentration (0.5mgl^{-1}) the sprouting percentage was maximum, and the duration for sprout initiation was minimum. This could be because cytokinins break apical

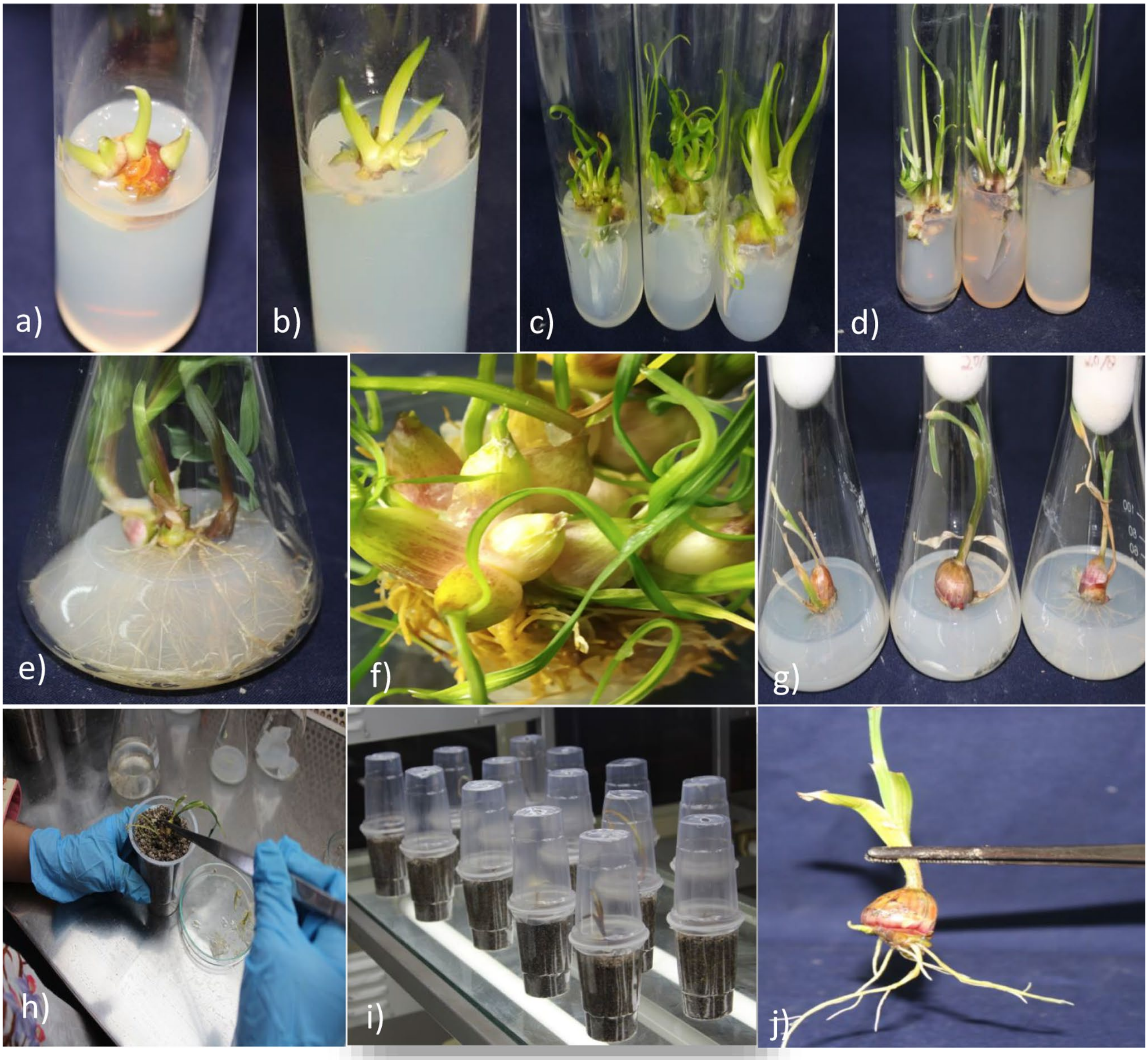

Fig. 3 Micropropagation protocol of *Gladiolus hybridus* Hort. cv "Red Majesty" from bud sprouting to cormlet production: **a** Bud sprouting; **b** Multiple shoot formation; **c**, **d** Shoot elongation; **e** Rhizogenesis and cormlet initiation; **f** Cormlet development; g) Cormlet maturation, **h** transfer into sterilized perlite + vermiculite (1:1) for hardening; **i** Hardening in jars; **j** Plantlet ready.for transfer to field

dominance and promote the growth of lateral buds. An earlier study (Beura and Singh 1998) also reports that higher levels of BAP (4.0 mg^{-1}) promoted secondary axillary bud production. Cytokinins at appropriate concentration levels enhance cell division and break apical dominance in gladiolus, promoting axillary shoot proliferation (Kumar et al. 2024). Since the cytokinin requirement is genotype dependent (Hussain et al. 2001), we developed a suitable cytokinin concentration for optimum shoot production in the gladiolus cultivar "Red Majesty". Twelve growth regulator combinations involving BAP at 0.5, 1.0, 1.5 and 2.0 mgl^{-1} in combination with GA_3 at two levels 1.0 and 2.0 mg^{-1} were used for bud sprouting (Table 1). Significantly higher sprouting percentage (91.25) and minimum days to sprouting (2.75) were achieved in treatment combination BAP (0.5mgl^{-1}) + GA_3 (2.0mgl^{-1}), while the maximum number of sprouts (4) per explant was obtained on BAP 2.0mgl^{-1}. An earlier study (Pathania et al. 2001) reported that BAP 2.0 to 4.0mgl^{-1} in combination with GA_3 was optimum for the culture establishment of CVS. 'Eurovision' and 'Wine and Roses'.

In the present study, the minimum days to sprouting were 2.75, while in earlier studies, it was reported to be 3.7 days (Priyakumari and Sheela 2006) and 7 days (Kumar et al. 2018). The media has a major effect on bud sprouting. (Kumar et al. 2018) used B5 media as well as MS media, with the former causing shoot bud initiation in 7–8 days while the latter it took 10–11 days. The present study shows that GA_3 at higher concentrations (in combination with a low concentration of BAP) significantly enhances sprouting percentage and induces early sprouting. GA_3 is active in breaking down the food reserve material by hydrolytic enzymes, which regulate the mobilisation of reserves, ultimately resulting in early sprouting.

The maximum number of sprouts per explant was 4.0 on MS supplemented with BAP 2.0mgl^{-1}, but the sprouting percentage was only 31. Therefore, overall, the best treatment for combined effect on bud sprouting (number of sprouts per explant and sprouting percentage) was MS + B_5 medium with BAP 2.0mgl^{-1} + GA_3 2mgl^{-1} showing 3.50 number of sprouts per explant and 61% sprouting percentage.

Shoot proliferation

Cormel sprouts (one week old) were used as explants for shoot multiplication. In an earlier study, cormel sprouts of medium size were found the most successful explants for in vitro mass propagation of Gladiolus (Noor-un-Nisa et al. 2013). Cytokinins effectively induce axillary shoot proliferation in Gladiolus (Shaheenuzzaman et al. 2011). We used BAP (1.0–3.0mgl^{-1}) along with kinetin (0.5–1.0mgl^{-1}) in combination with GA_3 (0.25–1 mgl^{-1}) for shoot proliferation studies. Maximum shoot number (20.50) was obtained on cytokinin combination BAP 3.0mgl^{-1} + kinetin 1.0mgl^{-1} (Fig. 3b,c,d). It is generally considered that a higher cytokinin-to-auxin ratio promotes shoot formation while a higher auxin-to-cytokinin ratio favours root differentiation. However, many studies on gladiolus have shown that it is not necessary to include both hormones in the medium for adventitious shoot formation or for axillary branching (Shaheenuzzaman et al. 2011), (Jala 2013). In the present study, a higher concentration of cytokinins increased the number of shoots per explant, which is consistent with an earlier study on gladiolus. In a similar study, (Grewal et al. 1995) reported a single shoot per explant on 1.0mgl^{-1} BAP and 14–20 shoot primordia at a higher (5.0mgl^{-1}) BAP concentration. In contrast, another study observed that low concentrations of cytokinin (BAP 1mgl^{-1}) produced more shoots (Aftab et al. 2008). The differences in the exogenous hormone requirement depend on the plant's endogenous levels, which vary from cultivar to cultivar in gladiolus (Dantu and Bhojwan 1992), (Hussain et al. 2001).

The present study achieved maximum shoot elongation on high cytokinin (BAP 3.0mgl^{-1} + Kn 0.5mgl^{-1}) and high GA_3 (1mgl^{-1}) concentration (Fig. 3d). It is consistent with a micropropagation study on gladiolus cv. Pusa Urmil reported that GA_3 in combination with cytokinins (and auxins) promoted the elongation of shoots (Singh et al. 2012). GA_3 has a major role in meristematic tissue development and it promotes vegetative growth by inducing active cell division in the apical meristem and the internodes. In the present study, GA_3 could have increased cell division and elongation in the intercalary meristem contributing to the elongation of shoots. Further, the minimum duration for shoot induction (2 days) was achieved on the same medium (BAP 3.0mgl^{-1} + Kn 0.5mgl^{-1} + GA_3 1.0mgl^{--}). In an earlier study, the medium that resulted in a minimum duration (2–3 days) for shoot induction had a higher concentration of BAP (2–4 mgl^{-1}), which is consistent with the present study (Priyakumari and Sheela 2006).

Corm development

In vitro studies on gladiolus show that different growth regulators including kinetin, gibberellin, abscisic acid and naphthalene acetic acid have been used for cormel development with varying degrees of success (Ginzburg and Ziv 1973), (Steinitz et al. 1991), (Palmer and Smith 1970), (Koda 1983), (Hussey and Stacey 1984). In one study, corm formation was reported on MS medium without the addition of growth regulators (Kumar et al. 2018).

In the present investigation, twelve growth regulator combinations consisting of IBA (0.5 mgl^{-1}) and CCC (1–2 mgl^{-1}) with different concentrations of sucrose (3, 6 and 9 g/l) were used to check the effect on corm development parameters like days to cormlet appearance, number of cormlets per explant, cormel size and cormel development percent. Persual of data (Table 3.) reveals that significantly higher number of cormels per explant (14) were obtained on combination IBA 0.5 mgl^{-1} + CCC 2.0 mgl^{-1} + sucrose 3% (Fig. 3f). In an earlier study on gladiolus the maximum number of cormels per shoot (5.8) was achieved on medium containing NAA (0.1 mgl^{-1}) and sucrose 3% (Jala 2013), while (Memon et al. 2014) obtained maximum number of cormels (12.06) on MS medium having sucrose 5% and IBA 1 mgl^{-1}. In the present study, minimum days to cormlet appearance (45 days), maximum cormlet development percentage (85%) and corm size (11 mm) were recorded on CCC 1.0 mgl^{-1} + sucrose 9%. (Goo and Kim 1994) reported that the highest (90%) in vitro cormel formation was obtained from the shoot base of gladiolus cv. Topaz on 9% sucrose. (Dantu and Bhojwani 1995) reported cormel formation on liquid MS medium supplemented with sucrose (6%) from 96% of shoots. (Emek 2007) reported corm formation on MS medium containing BAP (0.1 mgl^{-1}). In an earlier study, (Kim and Han 1993) reported that the use of CCC (chloromequat) was beneficial for cormlet formation in gladiolus. In potatoes, it has been reported that CCC promotes cormel initiation by reducing GA_3 biosynthesis and increasing tuberonic acid synthesis (Hussain et al. 2006). Other growth retardants like paclobutrazol too have been reported to be beneficial for in vitro cormel formation reported by (Nagaraju et al. 2002). Growth retardants with sucrose increase cormel size while decreasing leaf and stem growth (Nagaraju et al. 2002), which is consistent with the results in the present study. Higher sucrose levels (6–9%) have been found useful for the production of good-sized cormlets in gladiolus. In a study on potato, a higher level of sucrose caused increased osmolarity of the medium and caused stress to plants, thereby inducing maturity and tuber formation (Hussain et al. 2006). While Ahmad et al. reported cormel induction after 10–12 weeks on MS medium containing sucrose (6%)(Ahmad et al. 2000), in the present study, cormel induction was much earlier, i.e. within 6 weeks. The probable reason for earlier cormel induction could be due to the use of chlormequat chloride (cycocel; CCC) in addition to sucrose in our study. It was interesting to note that the use of auxin (IBA 0.5 mgl^{-1}) in combination with CCC (1.5 mgl^{-1}) enhanced the formation of shoot number, length and leaf number/shoot while decreasing the percentage of cormlet development and size (Table 4). This could perhaps be due to dynamics of reallocation of nutrients and change in carbon partitioning by the interaction between CCC (growth retardant) and IBA (auxin). It could also be the reason for having more cormels per explant (14) on IBA (0.5 mgl^{-1}) + CCC (2 mgl^{-1}) + 3% sucrose when a low concentration of sucrose (3%) was used. Although the number of cormels per explant is more, these do not develop into bigger-sized cormlets due to lack of sufficient sucrose.

In the present study, the cormels obtained after 9 weeks of culturing on MS media containing CCC were transferred to a half-strength MS medium containing 6% sucrose for increasing cormlet size. While there was an increase in the size of cormels (ranging between 2.75 and 11) obtained from all the nine treatment combinations, a maximum increase (11 mm) in cormel size occurred in cormels subcultured from CCC (1mgl^{-1}) + Sucrose (9%) (Table 4 and 5). After 4 weeks of transfer, the maximum size obtained was 22 mm (Fig. 3g). An earlier study confirmed the production of three types of corms from rooted shoots cultured in half-strength MS containing IBA (2.0mgl^{-1}) and sucrose, namely small (5–10 mm), medium (10–15 mm), and large (16–22 mm) (6 percent) (Sinha and Roy 2002). In a study on the variety White Friendship, the maximum size of cormel (2.8 to 3.2 mm) was obtained from rooted shoots cultured in MS medium containing sucrose (7%) and IBA (1.0mgl^{-1}). It has been reported that the in vitro produced cormels can be stored at 4 °C for 2–3 months and later sown in fields, where 80–90% germinate, out of which 20% flower successfully (Sinha and Roy 2002).

The maximum number of immature cormels (8–11 mm) per explant was 14 on MS supplemented with IBA (0.5mgl^{-1}) + CCC (2.0mgl^{-1}) + sucrose (3%), but the cormlet maturation percentage was only 56, therefore over all the best treatment for combined effect on cormlet development (cormlet maturation percentage and number of cormels) was observed in MS + B5 medium with IBA 0.5 mg^{-1} + CCC 2.0 mg^{-1} + sucrose 6% as we get 12 number of immature cormels per explant (8–11 mm) and 85 cormlet maturation percent of 18–22 mm size. The cormels were planted in small plastic jars containing perlite + vermiculite (1:1) and kept for hardening under 25 ± 2 °C in a walk-in plant growth chamber under 16/8 h photoperiod, and were occasionally exposed to ambient conditions under shade for one month (Fig. 3h, i). The plantlets were transplanted in the field after 6 weeks (Figs. 3, 4).

Conclusion

Many tissue culture companies use highly sophisticated plant tissue culture technologies to produce different floriculture crops of desired varieties/ clones for various commercial purposes in international markets. In the present study, a complete protocol for cormlet production of gladiolus was standardized. Growth regulator combinations positively affected all the 3 major stages, viz bud sprouting, shoot multiplication, and corm development of gladiolus. The highest sprouting percentage (91%) and the minimum number of days to sprouting (2 days) were obtained

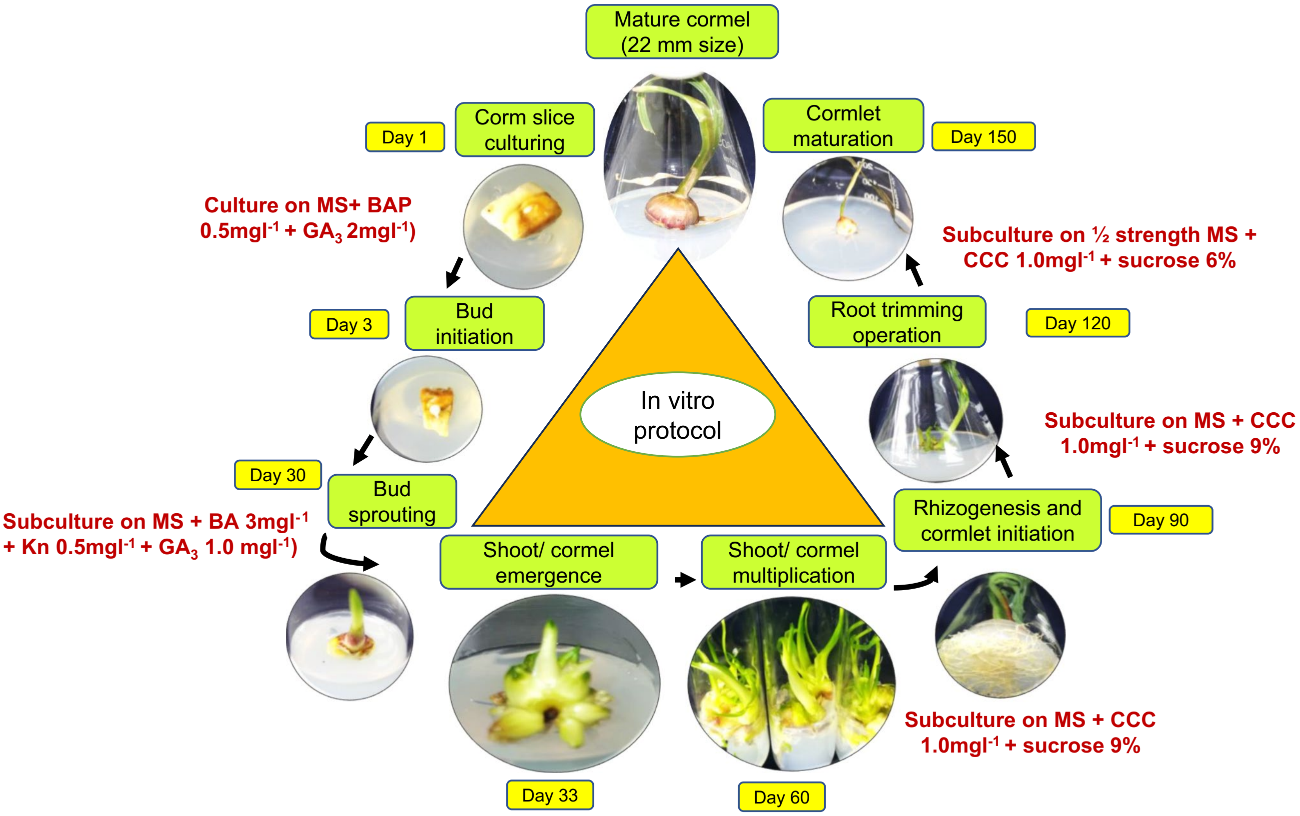

Fig. 4 Flow diagram of high efficiency micropropagation protocol for *Gladiolus hybridus* Hort. cv "Red Majesty"*:* Flow chart shows the culturing of explant on day 1, followed by 3 step subculturing on shoot/ cormel emergence medium (day 30); shoot/ cormel multiplication cum rhizogenesis medium (day 60); root trimming and subculturing on cormel maturation medium (day 120). After 150 days (5 months) 12–14 mature cormels (approx. 22 mm diameter) are produced from a single bud

on MS + B_5 medium having BAP (0.5 mg l^{-1}) + GA_3 (2.0 mg l^{-1}). In contrast, the maximum sprout number per explant (4) was obtained on MS + B_5 medium having BAP (2 mg l^{-1}). The maximum number of shoots per explant (15) was obtained on MS + B_5 medium having BAP (3.0 mgl^{-1}) + Kinetin (1.0 mgl^{-1}). Minimum days to shoot appearance (2), maximum shoot length (17 cm) and leaf number per shoot (9) were obtained on MS + B_5 medium having BAP (3 mgl^{-1}) + Kinetin (0.5mgl^{-1}) + GA_3 (1.0mgl^{-1}).

Under field conditions, the corm multiplication ratio of gladiolus ‘Red Majesty’ is reported as 1:2, while in the present protocol, a multiplication ratio of 14-fold could be achieved. The best medium for cormlet development was MS + B5 with IBA (0.5 mg^{-1}) + CCC (2.0 mg^{-1}) + sucrose (6%) with minimum days to cormlet appearance (45 days) and 85 percent cormlet maturation. In vitro propagation generates material free from viruses and other pathogens. Multiplying hybrid seeds from a few numbers in conventional breeding is tedious and time-consuming, and therefore, the present high-efficiency commercial micropropagation protocol shall overcome this constraint.

Acknowledgements The authors are highly grateful to the SKUAST-K for the support and facilities.

Author contributions M and AMH wrote the paper together with critical inputs from ITN. Wet lab experimental work was carried out by M under the supervision of AMH and ITN. All authors contributed to the article and approved the submitted version.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interests The authors have no conflict of interest and nothing to disclose.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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Industrial Crops & Products 222 (2024) 119521

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Microbial interactions modify saffron traits selectively and modulate immunity through adaptive antioxidative strategy: Organic cultivation modules should be trait and crop-specific

Samiullah Naik [a], Neha Bharti [a], Shakeel A. Mir [b], Firdous A. Nehvi [a], Amjad M. Husaini [a,*,1]

[a] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Jammu and Kashmir, India
[b] Division of Agricultural Statistics, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Jammu and Kashmir, India

ARTICLE INFO

Keywords:
Crocus sativus L.
Biofertilizers
PGPM
Azotobacter
Azospirillum
Microbial inoculation

ABSTRACT

Saffron (*Crocus sativus* L.) is a high-value, low-volume niche cash crop that has the unique ability to synthesize apocarotenoids. Since different biofertilizers are advocated for promoting plant health, we aimed to evaluate the interactions of commercially available microbial inoculants with this unique hysteranthous plant species. We studied the interactive effect of microbial inoculants on agronomical, physiological and biochemical aspects of Crocus growth, corm multiplication and apo-carotenoid biosynthesis. Significant differences in nutrient mobilization potential, induced systemic resistance, plant growth, corm, and stigma yield/quality were detected between the different biofertilizer combinations. The arbuscular mycorrhizal fungi (AMF) + phosphate-solubilizing bacteria (PSB) + potassium-solubilizing bacteria (KSB) combination was the most suitable for enhancing shoot biomass, root depth, and root biomass. Additionally, the average number of flowers per corm, stigma yield, and corm yield were greatest for the same combination. The highest multiplication rate of mother corms and a small corm index were recorded for plants inoculated with *Azospirillum* + PSB + KSB. Although *Azospirillum* caused maximum multiplication, the percentage of smaller daughter corms was greater, while the replacement of *Azospirillum* with AMF resulted in maximum percentage of larger corms. Plant health improved through AMF-mediated microbial interactions, which induced systemic resistance via enzymatic and nonenzymatic mechanisms and increased the uptake of P, K, Cu, Fe, Mn, and Zn. The interrelationship between plant and microbial consortium was complex, causing trait-specific qualitative and quantitative impacts on saffron attributes. Microbial inoculants increased nutrient uptake by saffron plants and triggered biochemical defence response by causing mild stress, making the plant more growth-responsive and better equipped for defence. Microbial interaction increased saffron yield and quality by favourably modulating plant metabolism, majorly overlapping with plant protection mechanisms. However, plant-microbial combinations in an organic production module are trait-specific and should be chosen carefully.

1. Introduction

Crocus sativus L. is a sterile triploid plant of the family Iridaceae. It is naturally propagated vegetatively by daughter corms that develop into mother corms (Kafi et al., 2018). The stigma 'saffron' of these monocot flowers is harvested, dried, and widely used as a spice to color food. Saffron fetches the highest price as a spice globally, at approximately US $ 1100–11000/kg, depending upon the country of its production (Husaini and Wani, 2020). Although leading traditional saffron producing countries are Iran, India, Spain, Italy, Greece, and Turkey, newer non-traditional areas are also being explored for its cultivation (like Afghanistan, USA, South Africa, Saudi Arabia etc) (Sheikh et al., 2023). It has countless biological properties, viz. anticancer, antimutagenic, antioxidant, antidepression, antiviral, antibacterial, immunomodulatory, neuroprotective, anesthetic effects and potential for use in epidemics like COVID-19 (Husaini et al., 2021; Ganai and Husaini, 2021). It

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* Corresponding author.
E-mail address: amjadhusaini@skuastkashmir.ac.in (A.M. Husaini).
[1] ORCID: 0000-0003-1293-3118

https://doi.org/10.1016/j.indcrop.2024.119521
Received 7 April 2024; Received in revised form 20 August 2024; Accepted 25 August 2024

is rich in proteins, vitamins (riboflavin and thiamine), potassium, iron, copper, zinc, sodium, and manganese (Moraga et al., 2009; Husaini and Wani, 2020). In addition to the apo-carotenoids crocin, picrocrocin and safranal, some studies have shown that saffron stigma and tepals contain other bioactive compounds, such as phenolic and flavonoid compounds, and exhibit antioxidant activity (Karimi et al., 2010; Baba et al., 2015; Sánchez-Vioque et al., 2012; Zeka et al., 2015; Tuberoso et al., 2016).

The ability of saffron to synthesize apocarotenoids is exceptional. Picrocrocin and crocin have been identified in only the stigma tissues of some *Crocus* species and a few other species, such as *Buddleja* (Liao et al., 1999) and *Gardenia* (Pfister et al., 1996). These peculiar qualities of saffron make it an exciting candidate for enhancing its production and quality. Agro-chemical free technologies are now being promoted for achieving the United Nations Sustainable Development Goals and a ‘greener’ green revolution (Husaini and Khurshid, 2021; Husaini and Sohail, 2023; Husaini and Sohail, 2024). Beneficial plant—microbe interactions are one among such technologies that improve the production and quality of many crops (Shabir et al., 2020). However, most of the studies on saffron have focused on isolating and characterizing microbes from the rhizosphere and cormosphere (Parray et al., 2013; Ambardar et al., 2016). Although these bacteria have plant growth-promoting traits *in vitro*, their success as ’biofertilizers’ under *ex vitro* field conditions has not been convincing, as saffron is cultivated in a monocropping system and has a long planting cycle of 5–6 years (Husaini et al., 2010a; Kafi et al., 2018). This could result in the loss of beneficial soil microflora of saffron fields and adversely affect soil health. Therefore, instead of isolating microbes from saffron corms and rhizospheres, a better approach is to study the effect of well-known commercially available microbial formulations on saffron growth, development and quality.

When plant growth-promoting microbes (PGPMs) are introduced into the soil, they face competition from indigenous microorganisms. The minimum population level of the initial inoculum needs to be maintained to promote plant growth (Persello-Cartieaux et al., 2003). However, the dynamics of the soil microbial population are complicated because inoculation with different microbial strain(s) affects the taxonomical or functional groups of autochthonous soil microorganisms in different ways (Malusà et al., 2016). Furthermore, the chemical compositions of the root exudates of different plant species vary (Walker et al., 2003; Weston et al., 2012; Moe, 2013), which leads to the establishment of diverse microbial communities in their rhizospheres. Biofertilizers that are suitable for one plant species may not be suitable for the other. Therefore, finding the most favorable plant—microbe interactions in a crop-specific manner is imperative. There is a need to identify the most suitable microbial consortium for saffron and to understand the effects of plant—microbe interactions on plant and soil health, stigma yield, and corm multiplication. These formulations should be readily available and commercially accessible to organic saffron farmers.

Considering the above, the present study was undertaken to determine the best possible combination of commercial formulations that could help produce saffron ’organically’ and ameliorate the adverse effects of stress faced by this crop under a changing climatic scenario (Husaini et al., 2013; Husaini, 2014). Apart from the control, four treatments were established; two constituents (PSB and KSB) were common but varied with respect to the third and/or fourth constituent. The objective was to identify a live formulation of commercially available beneficial microorganisms that could mobilize nutrients and improve soil health. Furthermore, the formulation should also help enhance disease resistance and withstand stress conditions (Husaini et al., 2012; Carbú et al., 2016; Shabir et al., 2020); therefore, we investigated the impact of microbial associations on the biochemical profile of saffron, particularly secondary metabolites associated with increased pigmentation and induced systemic resistance.

2. Materials and methods

2.1. Plant material and potting mixture

Fresh corms of saffron (*Crocus sativus* L.) were purchased during late summer season in the month of August from local farmers in Chandhara, Pampore, situated 34.1°N latitude, 74.90°E longitude altitude of 1650 m amsl (above mean sea level). The soil was collected from a saffron field in the same area (Kashmir’s traditional saffron growing region). The soil consisted of friable, loose, silty-clay loam soil belonging to the alfisol family (Ganai et al., 2000; Husaini et al., 2010a). Sterile sand was thoroughly mixed in equal proportions by volume (1:1) to prepare the potting mixture (pH 7.5). Healthy corms weighing 8–9 g were planted with one corm per ditch at a depth of 6–7 cm in trays (40×28×13 cm) on 23rd August and kept at ambient temperature under an open shelter at the Division of Plant Biotechnology, SKUAST-K, Shalimar. Three trays were used for each treatment, and each consisted of 4 corms planted in two rows with a planting distance of 15×20 cm. The nutritional status of the potting mixture was determined using the standard protocols described below.

2.2. Biofertilizer treatments

Liquid formulations of the microbial biofertilizers *Azotobacter* sp., *Azospirillum* sp., phosphate-solubilizing bacteria (PSB), potassium-solubilizing bacteria (KSB), and arbuscular mycorrhizal fungi (AMF) were procured from the Biofertilizer Production Unit, SKUAST-K and HiMedia Pvt., Ltd., New Delhi. The biofertilizers Azotobacter and Azospirillum consisted of multiple species. The liquid formulation of PSBs consisted of *Bacillus megaterium* and the *Bacillus subtilis strain DR2* (NCBI accession KP455653), while the liquid formulation of KSBs consisted of *Pseudomonas putida* and *Mesorrhizobium* sp. (NCBI accession MH503776) isolated from agricultural soils in Kashmir (Bashir et al., 2017; Bashir et al., 2018). The liquid formulation of AMF biofertilizer constituted four genera, *Glomus*, *Acaulospora*, *Scutellospora,* and *Gigaspora,* which were isolated from the apple orchard Kashmir, with *Glomus* being the predominant genus (Baba and Asif, 2018).

The treatments used were as follows: T_0 [sand: soil (1:1)], T_1 [sand: soil (1:1) + *Azotobacter* + PSB + KSB], T_2 [sand: soil (1:1) + *Azospirillum* + PSB + KSB], T_3 [sand: soil (1:1) + AMF + PSB + KSB], and T_4 [sand: soil (1:1) *Azotobacter* + *Azospirillum* + AMF + PSB + KSB]. *Azotobacter*, *Azospirillum*, PSB and KSB were inoculated at 25 ml/plant (of each inoculant) with a microbial population of CFU$\times 10^9$ cells/ml. AMF were inoculated at a density of 50 g per plant, resulting in a fungal population of 100 infective propagules per gram. The plants were reinoculated thrice with the biofertilizers at regular intervals of 30 days.

2.3. Physiological, growth and agronomic characteristics

Different plant physiological characteristics, viz. the shoot biomass (shoot dry weight), root biomass (root dry weight), root depth, days to 50 % flowering, and number of flowers per corm were determined as per the details mentioned below.

2.3.1. Shoot/root biomass and depth

Plantlets (triplicate) were taken from each treatment during the vegetative stage (21st of March) and separated into roots and shoots. The length of the longest root was measured with the help of a centimeter scale. These fractions were dried in a hot air oven at 65 ℃ for 48 h, after which the shoot and root biomasses (dry weight) were recorded using a digital balance.

2.3.2. Corm yield, indices, and multiplication rate

The the senesced plantlets (three per tray) were removed from each ditch after senescence stage (8th April) and corms were weighed to record the total yield. After weighing, the plants were categorized into three groups: (i) ‘big corms’ (corms weighing ≥ 10 g), (ii)’medium corms’ (corms weighing < 10 but ≥ 5 g), and (iii)’small corms’ (corms weighing < 5 g) (Agayev and Zarifi, 2009). The small corm index (%), medium corm index (%), big corm index (%), and corm multiplication rate (number of daughter corms produced per mother corm) were recorded.

2.3.3. *Days to 50 % flowering and no of flowers per corm*

The number of flowers emerging from each tray was noted daily during the saffron bloom period (Oct-Nov), and the total number of flowers was recorded for each treatment. The number of days to 50 % flowering under each treatment was recorded as the number of days required to achieve 50 % flowering. The ratio of the total number of flowers in each treatment to the total number of corms sown was used to estimate the number of flowers per corm.

2.3.4. *Stigma yield*

The flowers were harvested on the 3rd day after emergence during the bloom. Stigmas were separated immediately, and fresh weight (milligrams) was determined on a digital balance.

2.4. *Microbial count and AMF spore production*

Soil samples (20 g) were collected from the rhizosphere at 0, 25, 55, and 85 days, after which the effective microbial population in the treatments (before each inoculation) was determined. The soil was mixed thoroughly and sieved through a steel mesh with an 85 µm pore size. A small portion (1 g) of sieved soil was taken and homogenized in distilled water (pH 7). *Azotobacter, Azospirillum,* PSB, and KSB were isolated from the plants by the serial dilution plate method up to a dilution of 10^{-6} (Seeley Jr and VanDemark, 1962). These bacteria were subsequently plated on modified *Azotobacter* agar medium (*Azotobacter*), A+B medium (*Azospirillum*), Pikovskyas medium (PSB), and Aleksandrov agar medium (KSB). The media were procured from HiMedia™ Laboratories Pvt Ltd. (New Delhi, India) and were prepared according to the manufacturer’s instructions in the product technical notes M520 (Pikovskayas Agar), M371 (Azotobacter Agar), M518 (Azospirillum Medium), and M1996 (Aleksandrow Agar), which can be accessed through www.himedialabs.com. The plates were incubated for 3 days at 28°C ± 2°C, after which the colonies were counted using a colony counter.

The AMF spores were separated from the rhizosphere soil (50 g) by wet sieving and decanting (Gerdemann and Nicolson, 1963). These spores were isolated by sucrose gradient centrifugation (Daniels, 1982) and subsequently counted (Sylvia and Williams, 1992).

2.5. *Soil and plant nutrients*

For the soil analysis, samples (2–5 g per tray) were taken at flowering stage (1st week of November) and vegetative stage (3rd week of March) and treated following standard protocols. Macronutrients, viz. available nitrogen (Subbaiah, 1956), phosphorus (Olsen, 1954), potassium (Metson, 1957), organic carbon (Walkeley, 1947), and micronutrients viz. Mn, Zn, Fe, and Cu (Metson, 1957); boron (Berger and Truog, 1944); and molybdenum (Dass et al., 2014) were detected in all the treatment groups, including the control group, at both the saffron flowering and vegetative stages.

For the plant analysis, the whole plant samples (in triplicate) at the vegetative stage (3rd week of March) were oven-dried (at 65 ℃ for 48 h) and powdered using a mortar and pestle. The nutrient content was subsequently estimated using the standard protocols described above.

2.6. *Biochemical characteristics*

2.6.1. *Chlorophyll*

During the vegetative stage, 100 mg of fresh leaf (in triplicate) was ground in a mortar and processed. The absorbance was measured at 647 and 664 nm in a spectrophotometer, and the total chlorophyll, chlorophyll a, and chlorophyll *b* contents present in the extract were determined (Jeffrey and Humphrey, 1975).

2.6.2. *Total protein*

At the vegetative stage, 500 mg of fresh leaf (in triplicate) was macerated with a mortar and pestle. Total protein was estimated using the method given by (Waterborg, 2009).

2.6.3. *Phenol*

One gram of fresh leaf, taken in triplicate, was ground in a mortar and processed according to the protocol (Malick and Singh, 1980), after which the total phenol content was determined.

2.6.4. *Carotenoid*

Fresh leaf (1 g) and fresh stigma (500 mg) samples, taken in triplicate, were ground in a mortar and processed according to the protocol of (Liaaen-Jensen and Jensen, 1971) to determine the total carotenoid content.

2.6.5. *Apocarotenoid*

During the bloom, the flowers were harvested on the 3rd day after emergence. Stigma were separated immediately and oven-dried at 40°C ± 2°C for 8 h, and the moisture content was determined after drying the remaining stigma sample at 103 °C ± 2°C for 16 h. Fifty milligrams of each sample was used to estimate the crocin, picrocrocin and safranal contents by employing modified spectrophotometric procedures described in the ISO 3632 trade standard (ISO/TS 3632–2: 2010) (Caser et al., 2020). The parameters are the average of three replications of three measurements each.

2.6.6. *Proline*

Five hundred milligrams of fresh sample from each plant part was taken in triplicate and homogenized in 10 ml of 3 % sulfosalicylic acid, after which the proline concentration was determined using a standard protocol (Bates et al., 1973).

2.6.7. *Total sugars, reducing sugars, and Nonreducing sugars*

Plant samples from each plant part were oven-dried (80°C, 48 h) and powdered using a mortar and pestle. Dried leaf powder (50 mg) taken in triplicate was boiled in 10 ml of 80 % ethyl alcohol in a water bath. The homogenate was first cooled and then centrifuged at 600 rpm for 15 min. The supernatant was removed, and the volume was adjusted to 20 ml with 80 % ethyl alcohol. This extract was used for quantitative estimations of total sugars (Dubois et al., 1956), reducing sugars (Nelson, 1944), and nonreducing sugars (Loomis and Shull, 1937).

2.6.8. *Glycine betaine*

Five hundred milligrams of oven-dried (80°C, 48 h) finely ground plant material (triplicate) was mechanically shaken with 20 ml of deionized water for 24 h at 25°C, after which the glycine betaine concentration was estimated using a standard protocol (Grieve and Grattan, 1983).

2.6.9. *Catalase and superoxide dismutase*

For catalase (CAT) (Sinha, 1972), superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971), and (Asada et al., 1974), 200 mg of frozen sample (stored at −80°C) from each plant part was tested in triplicate, and the activities were determined using standard protocols.

2.6.10. *Ascorbate peroxidase (APOD)*

Ascorbate peroxidase (APOD) activity was determined according to previous methods (De Gara et al., 1997) using 700 mg of frozen plant sample (stored at −80°C) from each plant part, taken in triplicate.

2.6.11. *Ascorbate and glutathione*

Frozen samples of each plant part stored at −80°C (1 g each) were taken in triplicate, and the ascorbate content was determined by (Roe and Kuether, 1943) and (Shigeoka et al., 1979). Glutathione levels were determined from the frozen samples using standard protocols (Tietze, 1969; Griffith, 1980).

2.6.12. *Total antioxidant activity*

Tepals were harvested at the flowering stage, and corms were harvested at the vegetative stage and stored at −80°C until use. Frozen samples were frozen in triplicate (1 g each) and used to estimate total antioxidant activity (Prieto et al., 1999).

2.6.13. *Phenylalanine ammonium-lyase (PAL)*

Tepals and stigmas were harvested at the flowering stage, and root,

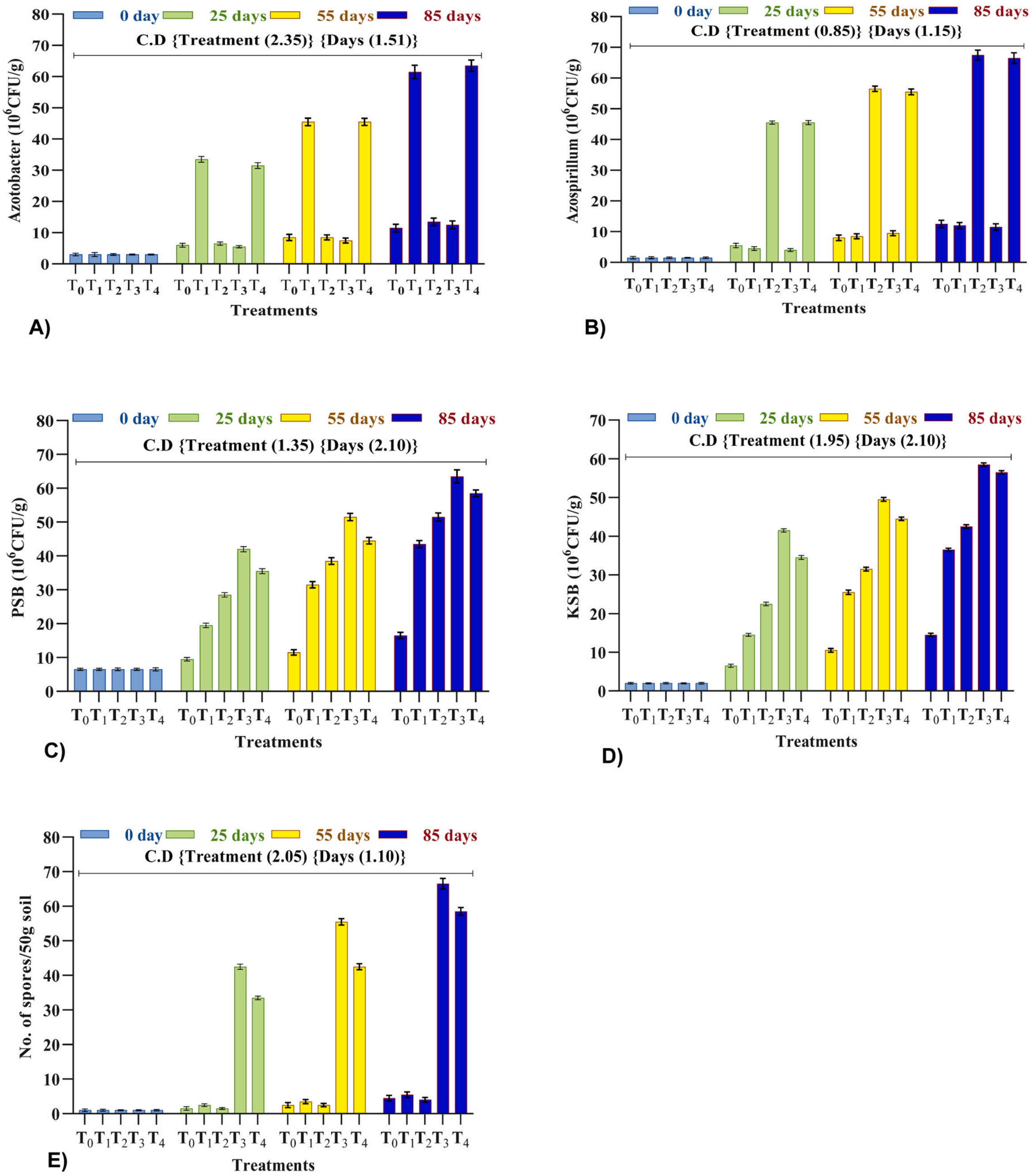

Fig. 1. Differential increase in the microbial count in the potting mixture under different treatments: The potting mixture was inoculated with 25 ml of plant material (with a microbial population of CFU×10^9 cells/ml) with each inoculant as per the respective treatment (T0, T1, T2, T3, and T4) on days 1, 30, 60 and 90, and the effect of each previous inoculation on the microbial count of *Azotobacter*, *Azospirillum*, PSB and KSB was recorded on the 25th, 55th, and 85th days. a) shows a significant increase in the population of *Azotobacter* under T1 and T4; b) shows a significant increase in *Azospirillum* under T2 and T4; c) shows a significant increase in PSB under T1, T2, T3 and T4; d) shows a significant increase in KSB under T1, T2, T3 and T4; e) shows an increase in KSB under inoculation with AMF @ 50 g per plant (with 100 infective propagules per gram) in accordance with the respective treatment (T0, T1, T2, T3, T4) on days 1, 30, 60 and 90; and d) shows the effect of each previous inoculation on the number of active AMF spores recorded on the 25th, 55th, and 85th days. A significant increase in the AMF population was recorded under T3 and T4. Treatment details: T_0 [sand: soil (1:1)], T_1 [sand: soil (1:1) + *Azotobacter* + PSB + KSB], T_2 [sand: soil (1:1) + *Azospirillum* + PSB + KSB], T_3 [sand: soil (1:1) + AMF + PSB + KSB], and T_4 [sand: soil (1:1) *Azotobacter* + *Azospirillum* + AMF + PSB + KSB].

corm, and leaf samples were collected at the vegetative stage and stored immediately in a deep freezer at −80°C. The frozen samples were analyzed in triplicate (1 g each) to estimate phenylalanine ammonium-lyase activity (Dickerson et al., 1984).

2.7. Statistical analysis

The experiment used a completely randomized block design (CRBD) (Haaland, 2020). Three trays were used for each treatment, and each consisted of 4 corms/ plants. The data on physiological, biochemical and morphological parameters (days to 50 % flowering, flowers per corm, stigma yield) noted at flowering stage are an average of ten replicates, while the data on remaining physiological, morphological, nutritional and biochemical parameters is an average of three. The total microbial count and soil parameter data were an average of three replicates. One-factor analysis of variance was performed for the soil and biochemical and physiological parameters, and two-factor analysis of variance was performed for the total microbial count via ANOVA procedures using Opstat software (http://14.139.232.166/opstat/) (Sheoran et al., 1998) and GraphPad software. Duncan's multiple range test (DMRT) was carried out to determine differences between means (Duncan, 1955).

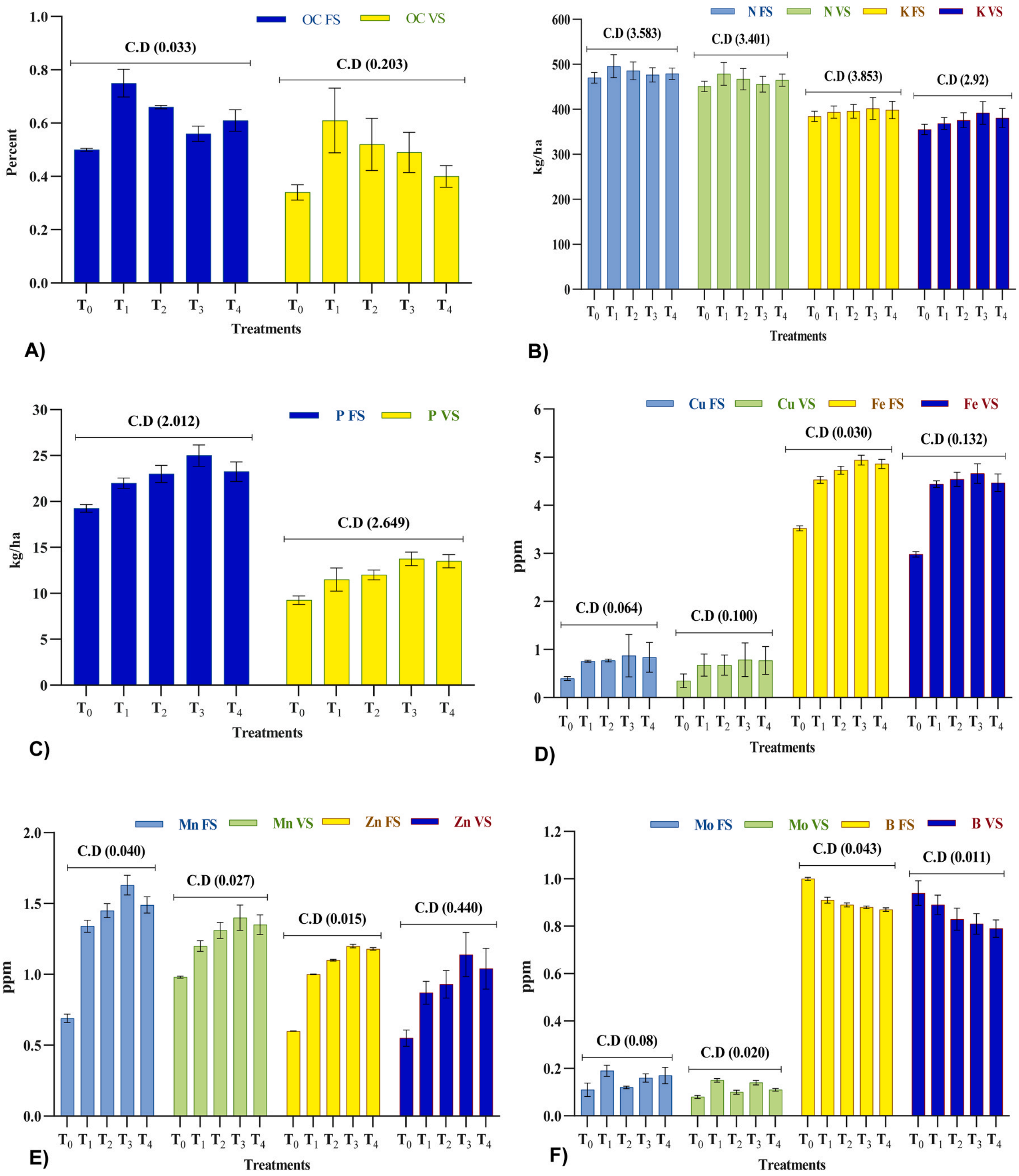

Fig. 2. Variation in the elemental profile of the potting mixture under different treatments. A total of 25 ml of the mixture was inoculated per plant (with a microbial population of CFU×10^9 cells/ml) with each inoculant as per the respective treatment (T0, T1, T2, T3, T4) on days 1, 30, 60 and 90, and the effects of the inoculations on the nutrient profile were determined at the flowering stage (FS) and vegetative stage (VS): a) Organic Carbon (OC); b) Nitrogen (N), potassium (K); c) Phosphorus (P); d) Copper (Cu), Iron (Fe); e) Manganese (Mn), zinc (Zn); and f) Molybdenum (Mo) and boron (B).

3. Results and discussion

Hereunder, we attempt to categorize the results under relevant subheadings and interpret them to derive outcomes so that the discussion becomes meaningful, cohesive and easier to follow.

3.1. *The effective microbial population in the rhizosphere significantly affects saffron growth and development*

The rhizosphere of saffron plants is a heterogeneous, continuous, and natural habitat in which different types of interactions occur between soil microbes and saffron roots. In the present study, we used beneficial microbes in different combinations to identify the best consortium and understand the nature of these interactions. These interactions are complex and host-plant specific. The microorganisms in the rhizosphere interact with many metabolites released by plant roots (Glick, 1995). These interactions could be positive, negative, or neutral (Filion et al., 1999).

The application of biofertilizers in the present study increased the counts of effective microbes, viz. *Azotobacter* sp., *Azospirillum* sp., PSB, KSB, and AMF in the soil (potting mixture), which was recorded at 0, 25, 55, and 85 days (Figs. 1–9). The native microbial population had no significant inhibitory influence on the microbial inoculants in the saffron planting soil, and there was no antagonistic effect between the inoculants. An incremental increase in the population of microbes was noted with time, reaching a maximum at 85 days after the first inoculation. Overall, the biofertilizers had a positive effect on the shoot growth parameters of saffron (Fig. 4). In an earlier report, *Bacillus subtilis* FZB24 inoculation of saffron corms under *ex vitro* conditions significantly increased leaf length, flower number per corm, and total stigma biomass and significantly decreased the time required for corms to sprout and the number of shoots (Sharaf-Eldin et al., 2008). (Nehvi et al., 2009) reported that inoculation with *Azospirillum, Azospirillum* and AMF increases saffron yield and is associated with an increased number of flowers, increased plant height, and increased leaf number. In the present study, biofertilizers positively affected both the root depth and the root biomass of saffron. The present study recorded the maximum shoot biomass (2.20 g) in plants inoculated with AMF + PSB + KSB (T_3). The AMF + PSB + KSB (T_3) combination was the most suitable for enhancing root depth and biomass, as it had more stout and longer roots than did the control (Fig. 4a, b, c). It is important to clarify that root length measures the length of the longest root and that root biomass is a better metric for representing overall saffron plant growth.

The integrated application of mycorrhizal fungi and organic and chemical fertilizers has been shown to significantly influence the overall production of saffron (Ghanbari et al., 2019). In the present study, biofertilizer treatments decreased the number of days to 50 % flowering and increased the number of flowers per corm in saffron (Figs. 4d, 5b).

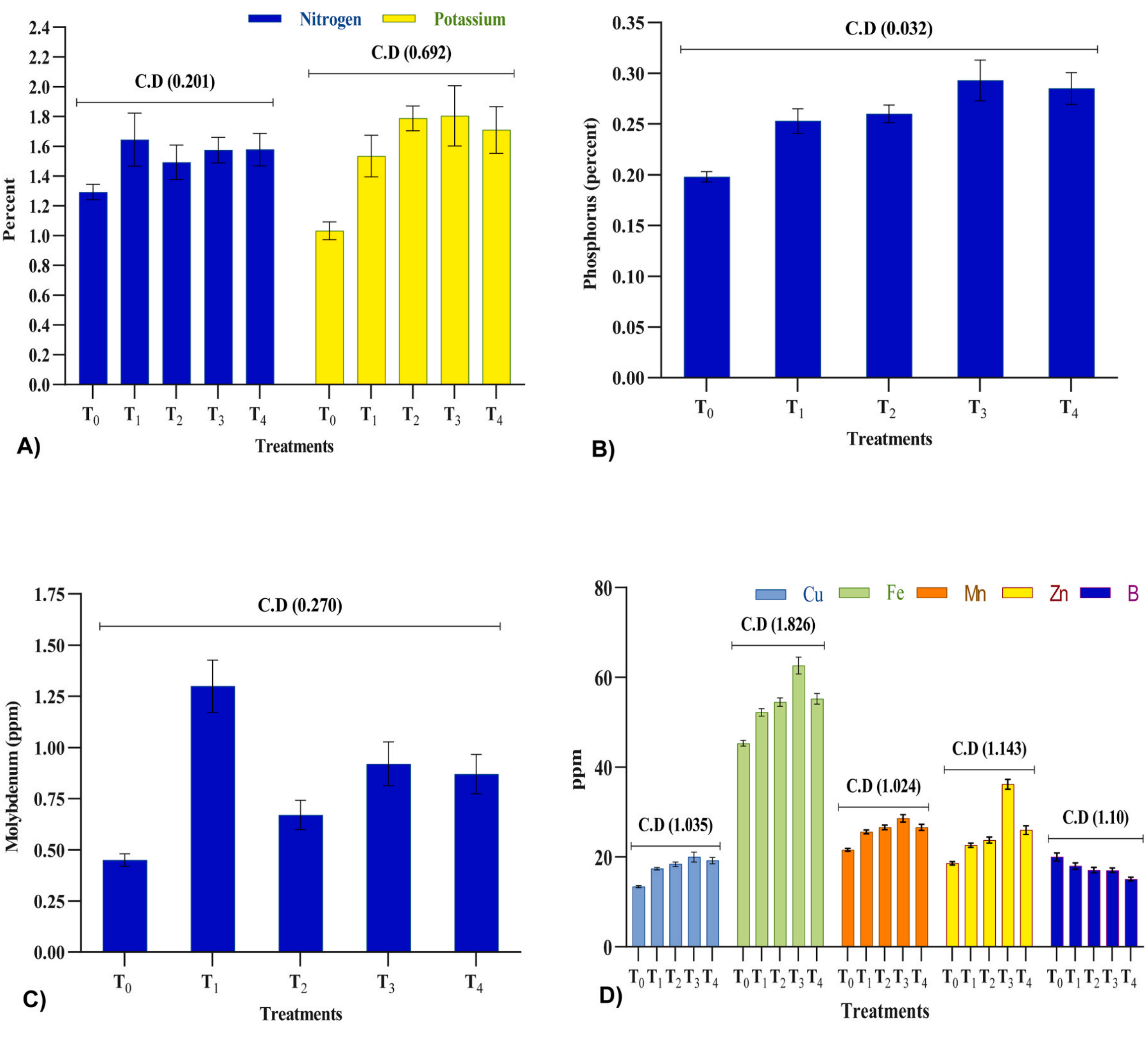

Fig. 3. Plant nutrient-elevation profile under different treatments: A total of 25 ml of pot mixture was inoculated per plant (with a microbial population of $\text{CFU}\times10^9$ cells/ml) with each inoculant as per the respective treatment (T0, T1, T2, T3, T4) on days 1, 30, 60 and 90, and the effect of inoculations on the plant nutrient profile was determined at the vegetative stage: a) N, K; b) P; c) Mo; d) Cu, Fe, Mn, Zn and B.

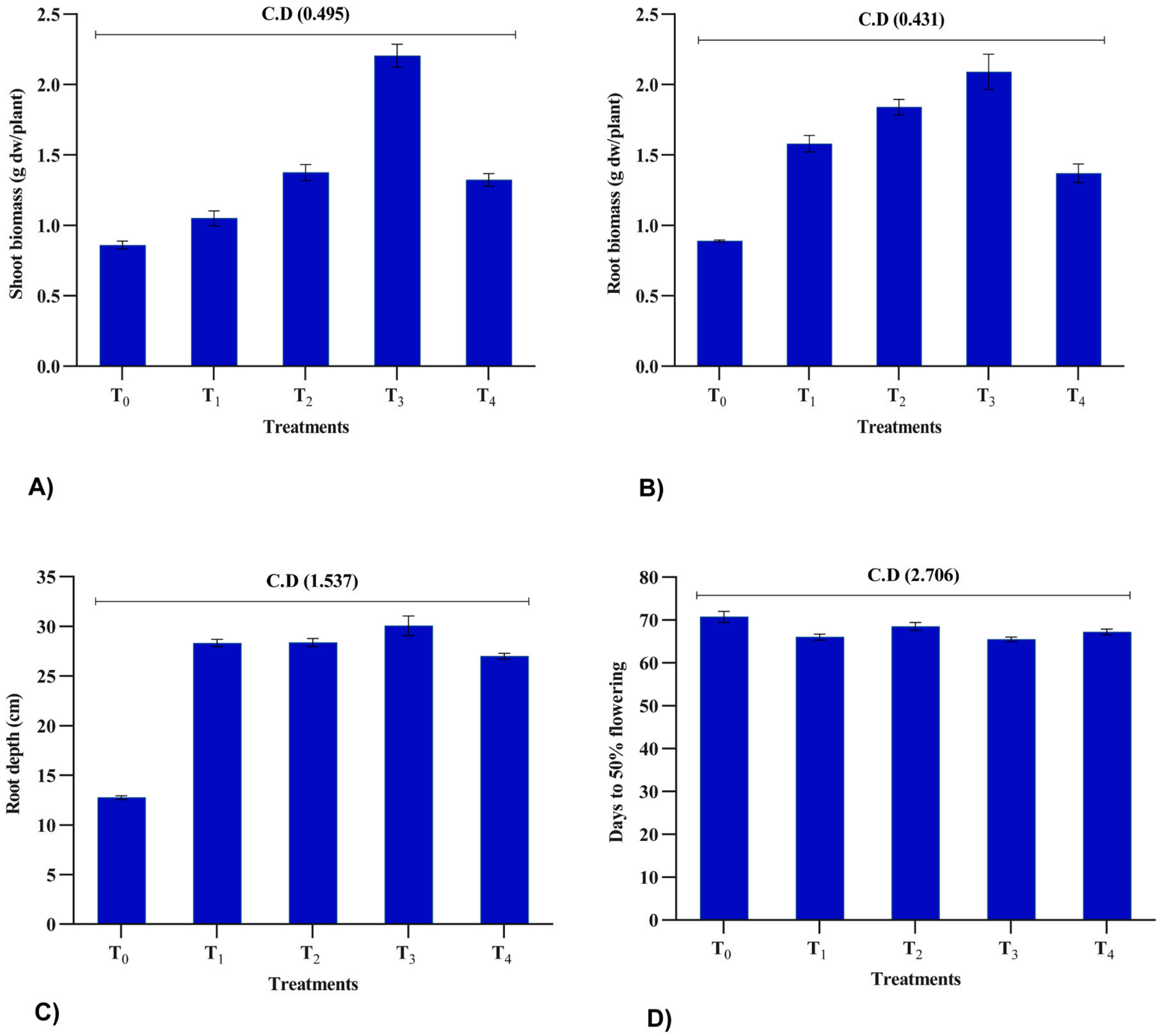

Fig. 4. Effects of different treatments on saffron plant growth and development: Plantlets were taken at the peak vegetative stage and oven-dried (at 65°C for 48 h) to record shoot and root biomass (dry weight). The number of days required to achieve 50 % flowering in each treatment was recorded as the number of days to 50 % flowering. The data are presented as a) shoot biomass, b) root biomass, c) root depth, and d) days to 50 % flowering.

The minimum number of days to 50 % flowering (65.50 days) and the maximum number of flowers per corm (3.5 flowers) were recorded for the plants inoculated with AMF + PSB + KSB (T_3). BF inoculation significantly enhanced the stigma yield, and the maximum increase of 33.33 % was achieved with AMF + PSB + KSB (T_3) inoculation. Additionally, the average number of flowers per corm (3.50 flowers), shoot biomass (2.20 g), root depth (30.05 cm), root biomass (2.09 g), stigma yield (0.03 g plant^{-1}), and corm yield (23.11 g) were greatest in T_3 (Figs. 4, 5).

The medium corm index ranged from 25.03 % to 30.58 %, with the maximum medium corm index occurring in T_4 (30.58 %) and the minimum occurring in T_2 (25.03 %). The maximum large corm index was recorded in T_0 (38.29 %), while T_1 plants had the lowest large corm index (24.10 %). The maximum small corm index was 48.53 % (T_2), and the minimum was 31.66 % (T_0). The maximum corm yield was recorded in T_3 (23.11 g), while the minimum yield (11.76 g) was recorded in T_0. The highest multiplication rate of mother corms (4.18) and small corm index (48.53 %) were recorded for plants inoculated with *Azospirillum* + PSB + KSB (T_2), while the lowest multiplication rate (2.62) was in control (T_0). The maximum corm yield (23.11 g) and maximum large corm index (35.74 %) was recorded for plants inoculated with AMF + PSB + KSB (T_3). Taken together, the results for the corm indices and multiplication showed that T_2 caused maximum multiplication but resulted in a greater percentage of small sized corms, while T_3 and T_4 resulted in greater percentages of big and medium sized corms, respectively (Fig. 5).

Azotobacter-, *Azospirillum*-, PSB-, and KSB-containing microbial consortia significantly improved saffron plant growth (Fig. 4 & 5). The positive results of PSBs can be attributed to their well-established role in the secretion of organic acids and phosphates to solubilize insoluble phosphate into soluble forms. *Azotobacter* and *Azospirillum* are known to secrete vitamin B complex, naphthalene acetic acid, indole acetic acid, cytokinin, and gibberellins (Mahanty et al., 2017). Inorganic phosphorus solubilization occurs through the action of low-molecular-weight organic acids such as gluconic and citric acids (Glick, 2012). The hydroxyl and carboxyl groups in these organic acids chelate the cations bound to phosphate, converting insoluble phosphorous to its soluble form. Organic phosphorus is mineralized by synthesizing different phosphatases that catalyze the hydrolysis of phosphoric esters (Acosta-Martínez and Ali Tabatabai, 2011). Both phosphate solubilization and mineralization can occur in the same bacterial strain (Pereira and Castro, 2014).

Potassium-solubilizing bacteria (KSBs) also positively affect saffron growth and economic characteristics (Figs. 4, 5). KSBs can solubilize potassium rock through the production and secretion of organic acids. KSBs also play an important role in maintaining soil structure by contributing to the formation and stabilization of water-stable soil aggregates (Han and Lee, 2006).

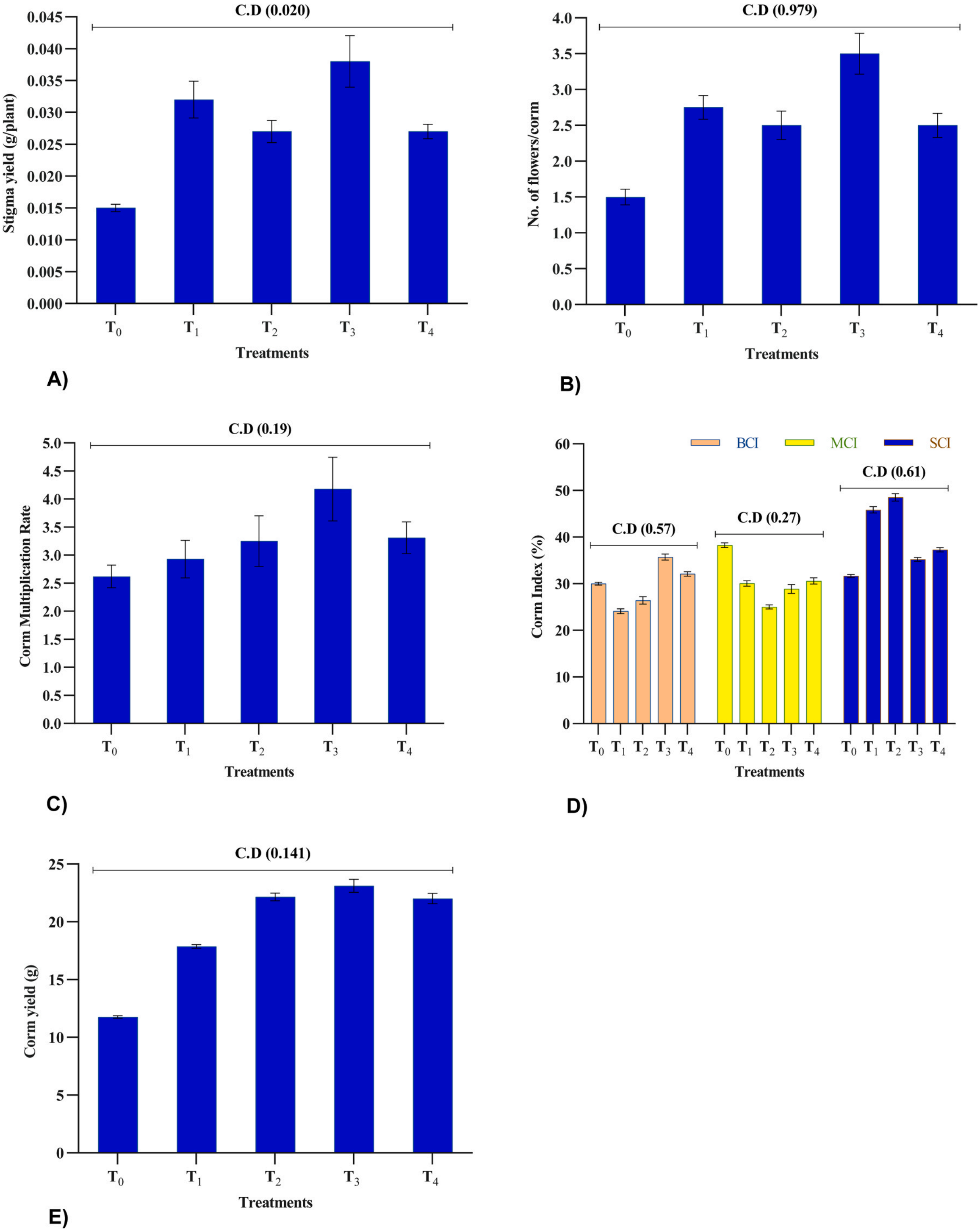

Fig. 5. Effects of different treatments on saffron agronomic parameters: a) stigma yield (fresh weight), b) number of flowers per corm, c) multiplication rate, d) large corm index (BCI), medium corm index (MCI), small corm index (SCI), and e) corm yield per ditch.

Overall, biofertilizer treatment T_3 (AMF + PSB + KSB) had the greatest effect on the general growth characteristics (shoot and root growth, increased number of flowers, early flowering) and economic characteristics (maximum stigma, corm yield and big corm index) of saffron. AMF in T_3 (AMF + PSB + KSB) had a significantly greater positive influence on saffron growth (Fig. 4 & 5). These factors alleviate the limitation of plant growth caused by an inadequate nutrient supply and improve tolerance to biotic and abiotic stresses (Todeschini et al., 2018). Earlier reports indicate that well-established AMF colonization of saffron roots, either alone or with plant growth-promoting bacteria, improves plant growth characteristics. This results in increased corm phosphorus content, chlorophyll content, corm weight, and leaf matter and increased soil nitrogen and phosphorus assimilation (D'Agostino et al., 2009; Mohebi-Anabat et al., 2015; Lone et al., 2016). The PGPM consortium (T_3) could be more efficient due to the different mechanisms of action of the various microorganisms present, which sometimes overlap with plant protection mechanisms (Vassilev et al., 2001; Vassilev et al., 2006).

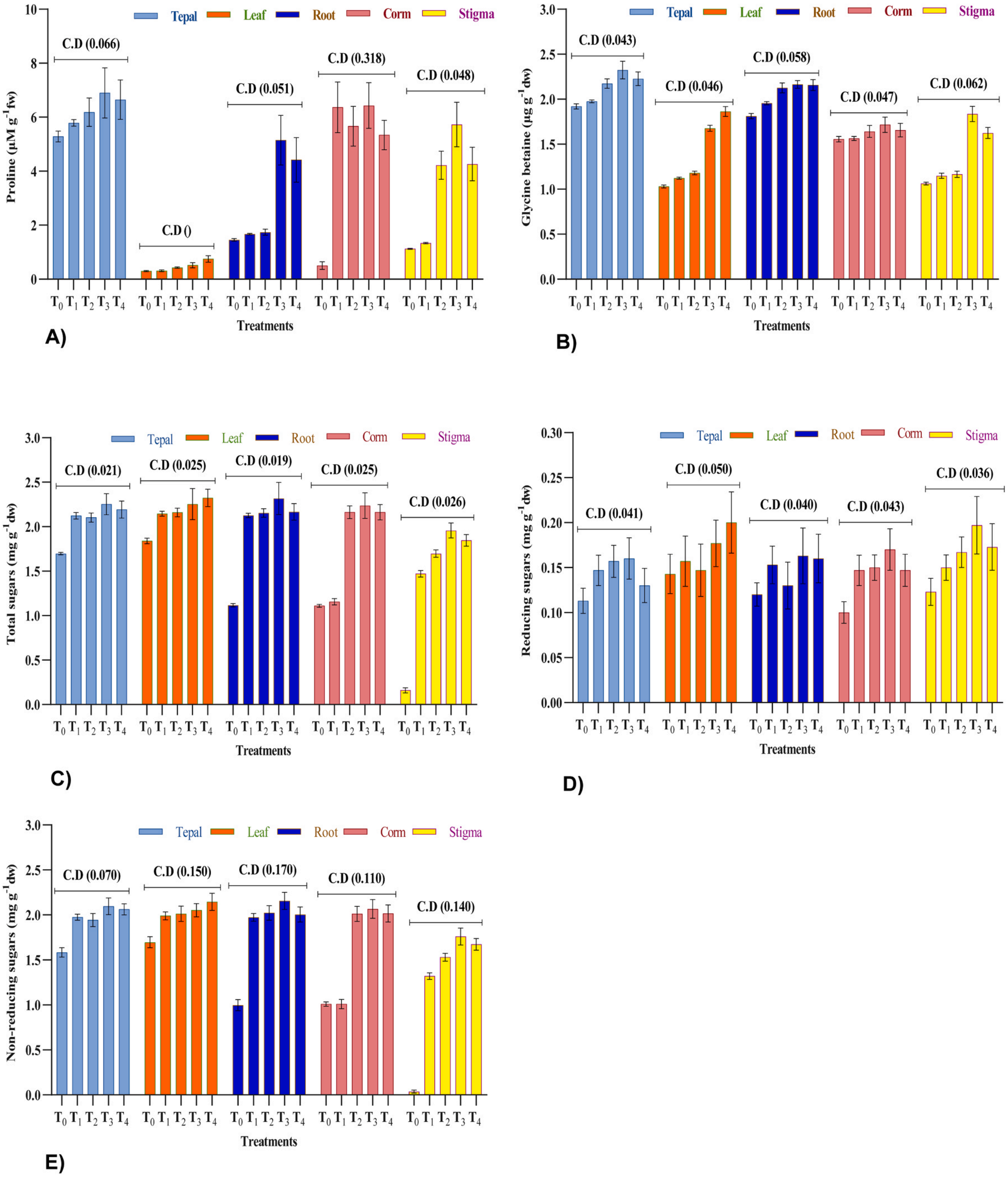

Fig. 6. Effects of biofertilizer treatments on sugars and osmolytes in different plant parts of saffron. The quantity of each plant part was determined in triplicate. For proline estimation, (a) the samples were immediately processed fresh, while the rest of the samples were oven-dried (80°C, 48 h) and processed to determine b) glycine betaine, c) total sugars, d) reducing sugars, and e) nonreducing sugars.

3.2. Microbes influence nutrient availability and mobilization in saffron differentially

The beneficial plant-microorganism interactions in the rhizosphere are the primary determinants of plant health and soil fertility (Jeffries et al., 2003). *Azotobacter* sp. are known to improve the bioavailability of nitrogen to plants. *Azospirillum* is an associative microaerophilic nitrogen fixer commonly associated with the roots of higher plants, especially C4 crops such as maize, sorghum, sugarcane, and pearl millet. (Mahanty et al., 2017). Its low energy requirement, high nitrogen fixation capacity, abundant establishment in the roots of taller plants, and tolerance to high soil temperature (30–40°C) are responsible for its wide suitability (Husaini et al., 2022). Both of these bacteria are known to increase the uptake of the mineral elements N, P, and K (Mahanty et al., 2017).

Bacteria promote organic matter decomposition and accelerate the mineral nutrition cycle, thereby maintaining and improving soil fertility (Pang et al., 2009; Yang et al., 2015). In the present study, the organic carbon (OC), nitrogen (N), available potassium (K), available phosphorus (P), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), molybdenum (Mo), and boron (B) contents of the potting mixture at the flowering and vegetative stages significantly differed among the treatments (Fig. 2). AMF fungi have been shown to cause more than twofold increases in the availability of less mobile nutrients (P, Ca, S, Zn, Mg, and Cu) from the rhizosphere (Manjunath et al., 1981; Barea, 1991). Our

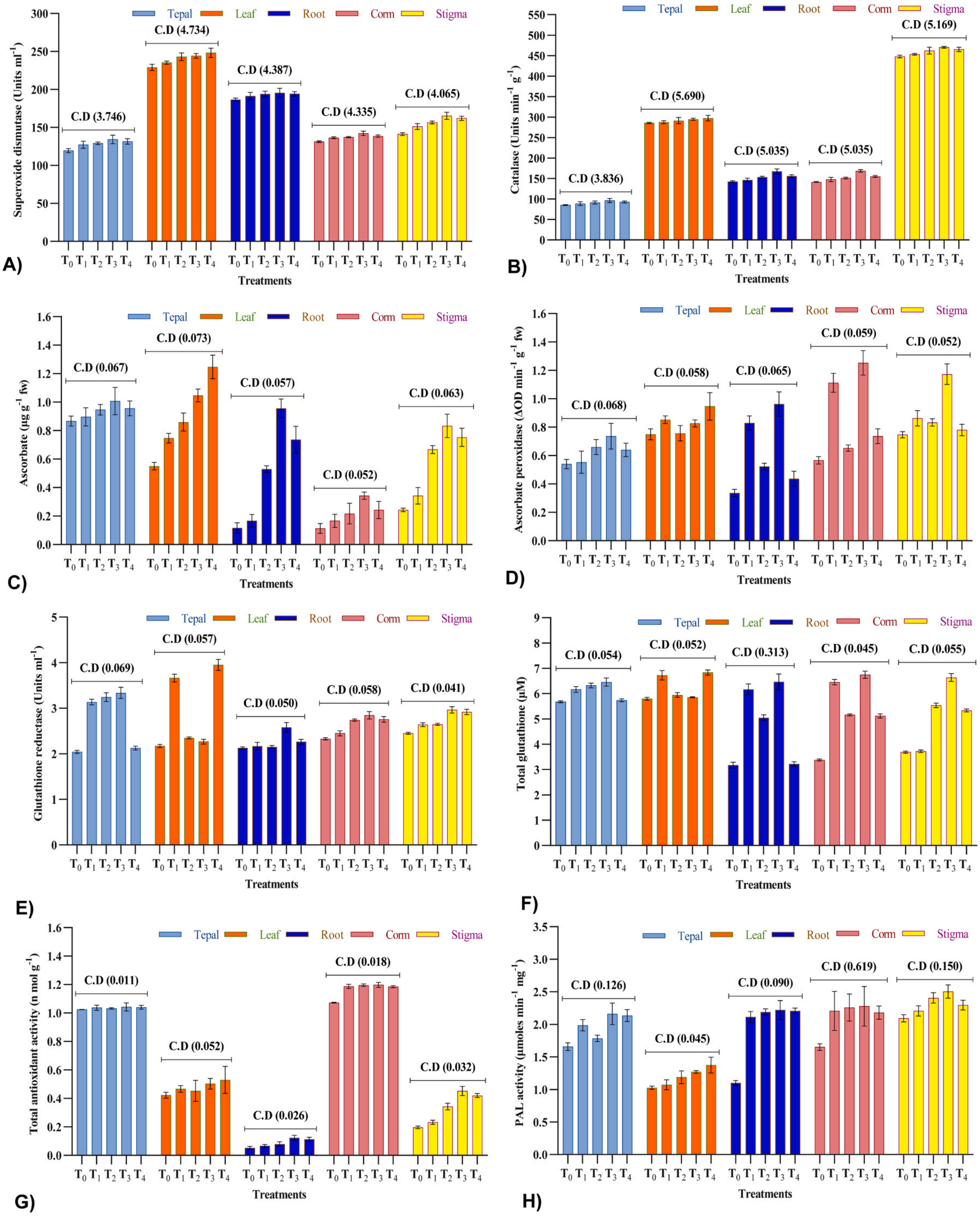

Fig. 7. Effect of different treatments on antioxidative and defense enzymes: Adequate fresh sample of each plant part was taken in triplicate, stored at −80℃ till use, and then processed to determine a) super oxide dismutase, b) catalase, c) ascorbate, d) ascorbate peroxidase, e) total glutathione, f) glutathione reductase, g) total antioxidant activity, h) PAL enzyme activity.

results support the role of AMF in increasing micronutrient availability in soil. It has been reported earlier that the application of *Azotobacter* along with AMF and the recommended fertilizer dose results in high soil organic carbon status and greater phosphorus uptake in onion (Talwar et al., 2017). In the present study, the soil OC, N and Mo contents (at both the flowering and vegetative stages) increased significantly in T_1 (*Azotobacter*+PSB+KSB). In contrast, the P, K, Cu, Fe, Mn, and Zn contents were significantly greater in T_3 (AMF + PSB + KSB). Microbial inoculation had a significant effect on plant macronutrient (N, P, K) and micronutrient (Cu, Fe, Mn, Zn, Mo, B) uptake at the vegetative stage (Fig. 3). The maximum increase in the uptake of P, K, Cu, Fe, Mn, and Zn was found in $T_{3,}$ while the maximum N and Mo uptake was observed in $T_{1.}$ In addition to N, *Azotobacter* seems to cause an increase in the availability and uptake of Mo (Figs. 2f, 3c). *Azotobacter* not only provides N_2 but also produces a variety of growth-promoting substances, such as indole acetic acid (IAA), gibberellins, and vitamin B (Martinez-Toledo et al., 1988; Torres-Rubio et al., 2000; Ahmad et al., 2005; Teng et al., 2006); and antimicrobial and antifungal substances (Verma et al., 2001; Mali and Bodhankar, 2009).

The data in the present study showed that biofertilizers affect the

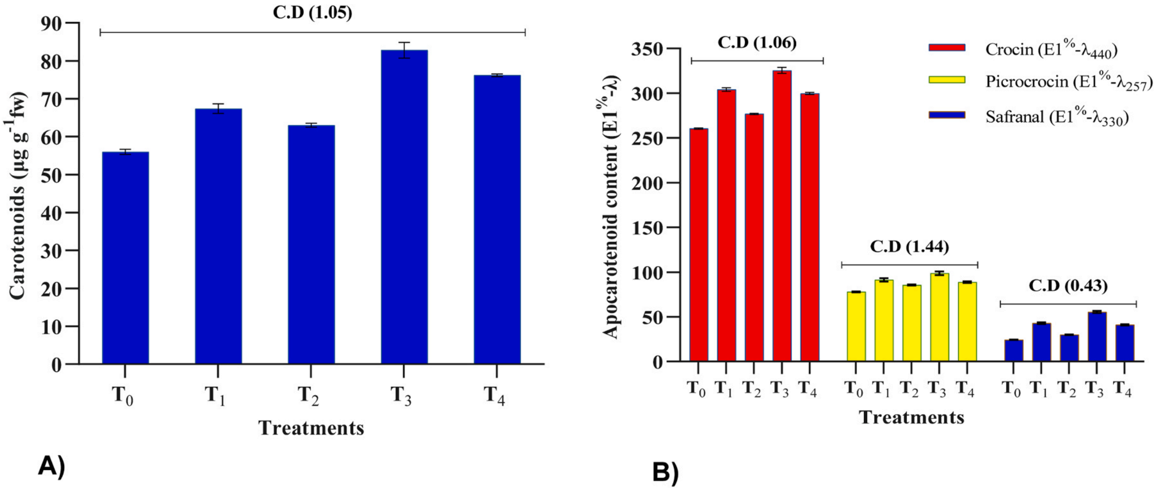

Fig. 8. Effects of different biofertilizers on pigment accumulation in saffron stigma: Fresh stigma samples were used to measure a) carotenoid content, while oven-dried stigmas were used to determine b) apocarotenoid content.

microbial content of soils and nutrient uptake in saffron (Figs. 2, 3). Surprisingly, there was a decrease in the B content in the plant tissue in all the biofertilizer treatments, perhaps owing to the immobilization of B by the microbial inoculants for their own metabolism.

3.3. Microbial interactions trigger stress response mechanisms and induce systemic resistance

The effects of biofertilizer treatments on the proline, glycine-betaine, and sugar contents in all parts of the saffron plants were also studied (Fig. 6). The proline content in the leaves was significantly greater in the T_{3}- and T_4-inoculated plants, with a maximum proline content (0.75 μM g^{-1}) occurring in T_4. This increase was 40.30 % compared to that of the non-inoculated plants (Fig. 6a). The mean values were much greater for the other saffron plant parts, viz., tepal, root, corm, and stigma. Similarly, compared with those in the control treatment, the proline content in the T_3 (AMF + PSB + KSB) and T_4 (*Azotobacter* + *Azospirillum* + AMF + PSB + KSB) treatments significantly increased across all the plant parts. There was a significant increase in glycine betaine content with microbial inoculation in all the plant parts in all the treatments. However, T_3 and T_4 caused the maximum increase in glycine betaine, while T_1 and T_2 caused only a marginal increase (Fig. 6b). Additionally, there was a significant increase in the sugar content in all the plant parts of the microbially treated saffron plants.

Phenylalanine ammonium-lyase (PAL) plays a pivotal role in phenolic compound synthesis and is used to monitor and predict defense responses. Considering its role in protecting plants against oxidative stress, PAL activity was studied in saffron plants. The maximum PAL activity was recorded in the stigma, followed by the corm, root, tepal, and leaf (in decreasing order) (Fig. 7g, h). Biofertilizer treatments cause a maximum increase in PAL activity in subterranean parts, such as roots and corms, possibly leading to induced systemic resistance (ISR). Many reports emphasize the correlation between an increase in PAL activity in response to different stimuli. According to many studies on other crops, enhanced PAL activity in plant tissues is positively associated with ISR and plant disease suppression (Chandrasekaran et al., 2017; Li et al., 2012; Li et al., 2015; (Mauch-Mani and Slusarenko, 1996; Prathuangwong and Buensanteai, 2007). PAL is among the many enzymes that increase resistance against *Xanthomonas oryzae* pv. oryzae in rice (Song et al., 2016; Yasmin et al., 2016; Solekha et al., 2020). Similarly, in mung bean, ISR was developed against *Macrophomina phaseolina* through enhanced PAL levels by using a combination of bacteria, *Pseudomonas putida* CRN-09, and *Bacillus subtilis* CRN-16 (Sharma et al., 2018). The phenylpropanoid pathway is activated in *Prosopis glandulosa* plants exposed to cadmium and copper (González-Mendoza et al., 2018). It acts as a source of nonenzymatic antioxidants that protect against oxidative stress. In cucumber and rice, ISR mediated by PGPR is associated with enhanced PAL enzyme activity (Chen et al., 2000 ; Meena et al., 2014).

Several studies have shown that Bacillus sp. induces plant systemic resistance, which is associated with the activation of plant defense mechanisms (Ferraz et al., 2015; Jetiyanon, 2007; Kloepper et al., 2004; Li et al., 2015; Van Loon, 2007). In the present study, all the microbial combinations enhanced the PAL activity in the saffron plant parts, with the greatest increase occurring in the corms and roots (Fig. 7**h**). Among the treatments, inoculation with AMF + PSB + KSB (T_3) resulted in the highest PAL enzyme activity in the stigma (2.50 μmoles $min^{-1}mg^{-1}$), followed by that in the corm (2.27 μmoles $min^{-1}mg^{-1}$), root (2.21 μmoles $min^{-1}mg^{-1}$), and tepal (2.16 μmoles $min^{-1}mg^{-1}$). This is a very useful outcome because saffron is susceptible to many soil-borne diseases (Ahmed and Husaini, 2021; Husaini, 2014; Husaini et al., 2010a; Kafi et al., 2018); therefore, microbial inoculation can be instrumental in enhancing tolerance mediated by PAL activity.

Furthermore, increased PAL activity is associated with many medicinal properties in plants. *Rhodotorula glutinis* (a yeast) and plants naturally growing under salt stress have high PAL production/activity and are therefore used in the food and pharmaceutical industries as good sources of natural antioxidants (Kot et al., 2016; Şirin and Aslım, 2019). Our findings in saffron suggest that microbial inoculation can enhance PAL activity and antioxidant potential in other medicinal plants of commercial value.

The findings of the present study indicate that while microbial inoculants increase nutrient uptake by saffron plants, they may also cause mild stress to plants, triggering biochemical defense (Fig. 6c, d, e). These biochemical changes increase plant responsiveness to growth and help plants become better equipped for defense.

3.4. Biofertilizer application increased ascorbate, glutathione contents and antioxidant activities

Plants have developed antioxidant enzymes such as catalase (CAT), glutathione reductase (GR), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), and nonenzymatic ROS scavengers such as glutathione, tocopherols, flavonoids, phenolics, ascorbic acid, carotenoids, lycopenes and alkaloids to reduce oxidative damage due to stress (Caverzan et al., 2012). The present study showed that biofertilizers enhance the stress preparedness of saffron by activating enzymatic and nonenzymatic antioxidant responses. However, the

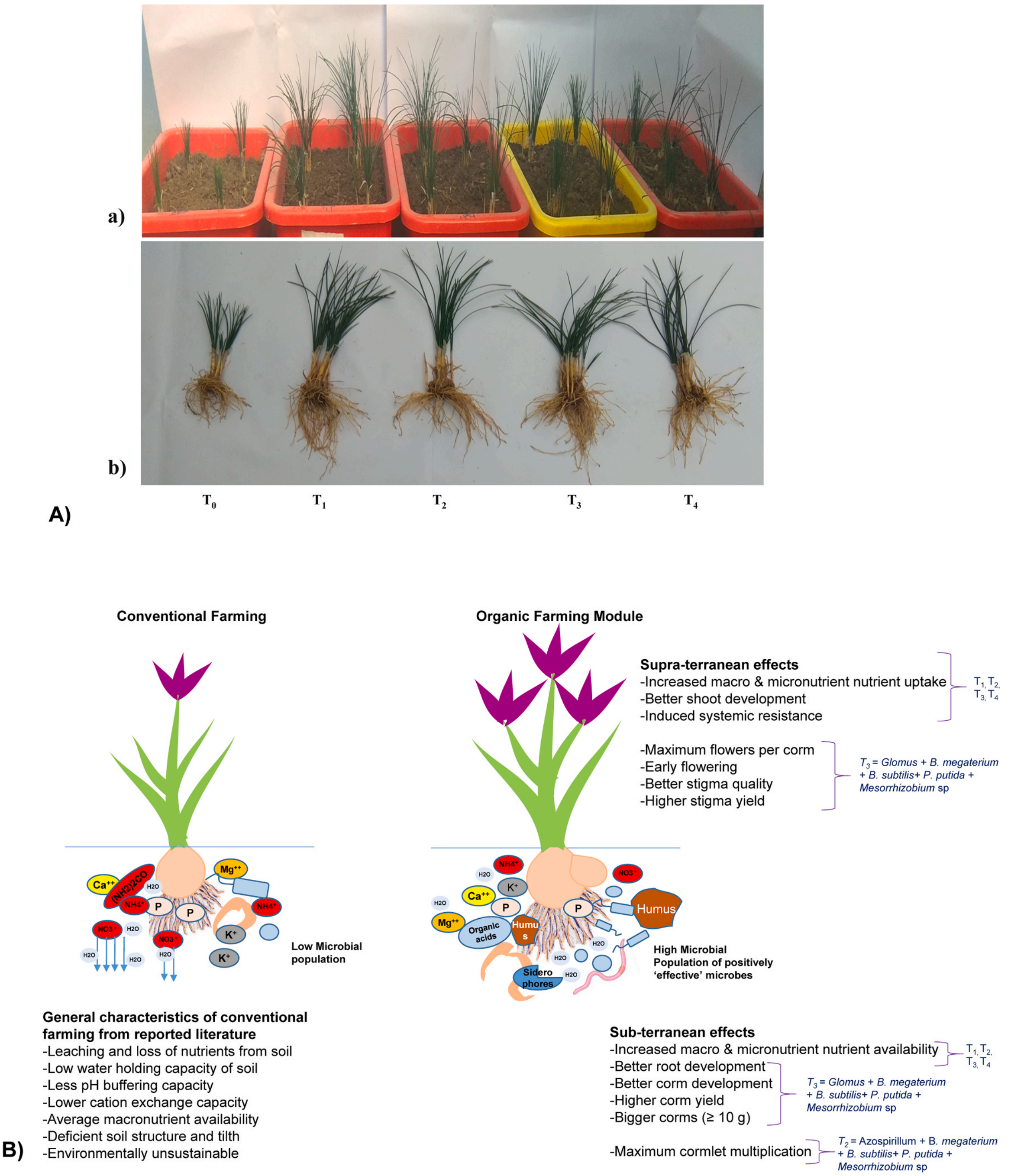

Fig. 9. A) Growth and development of saffron plants under different treatments: a) In trays; b) uprooted plants at vegetative stage (3rd week of march). Treatment details: T_0 [sand: soil (1:1)], T_1 [sand: soil (1:1) + *Azotobacter* + PSB + KSB], T_2 [sand: soil (1:1) + *Azospirillum* + PSB + KSB], T_3 [sand: soil (1:1) + AMF + PSB + KSB], and T_4 [sand: soil (1:1) *Azotobacter* + *Azospirillum* + AMF + PSB + KSB]. B) A schematic comparison of conventional (control) and trait-specific organic farming module of saffron: Besides some well known benefits of organic farming (viz. slow and sustained release of nutrients, high water holding capacity, better pH buffering capacity, higher cation exchange capacity, improvement in soil structure and tilth, safety for human consumption), the present study revealed that organic carbon in soil and sugar in all parts of plants increased under all microbial treatments (T_1, T_2, T_3, T_4) as compared to control. Availability of all major and micro nutrients increased through all microbial treatments (T_1, T_2, T_3, T_4), and the uptake was higher in plants cultivated under organic conditions (T_1, T_2, T_3, T_4). Enzymatic as well as non-enzymatic antioxidant activity increased under organic farming, thereby causing induction of systemic resistance (ISR) in plants (T_1, T_2, T_3, T_4). However, there were differences between these organic treatments with respect to the 'magnitude' of response/ effect (see Figs. 2–8). Moreover, some treatments were better than others for certain plant traits (see T_2 and T_3). AMF fungi promoted root and shoot biomass in saffron, especially when used in combination with PSB and KSB. In addition to nitrogen, *Azotobacter* sp. also increased the availability and uptake of molybdenum.

activities of the antioxidant enzymes varied between the treatments. For example, biofertilizer application caused a mild increase in SOD activity in all the treatments but had no significant effect on catalase activity. Among the saffron plant parts (tepal, root, corm, stigma and leaf), SOD activity was highest in the leaves (Fig. 7a), while catalase activity was greatest in the stigma (Fig. 7b). In contrast to those of SOD and catalase, the ascorbate content and ascorbate peroxidase activity varied significantly in response to biofertilizer application. In the roots, stigma corms and tepals, the maximum values were recorded in T_3; in the leaves, the maximum concentration was in T_4 (Fig. 7c, d). Microbial inoculation caused a sharp increase in glutathione and glutathione reductase activity in nearly all the treatments and in all the saffron plant parts (Fig. 7e,f).

The total antioxidant activity estimated by the phosphomolybdenum method was significantly different among the treatments, especially in the stigma, where the maximum antioxidant activity was observed in treatment T_3. Corms showed greater antioxidant activity than tepals. The antioxidant activity of the saffron corms inoculated with AMF + PSB + KSB (T_3) was greater (1.198 nmol g^{-1}) than that of the noninoculated plants (1.073 nmol g^{-1}) (Fig. 7g). These natural antioxidants are multifunctional (Kumar et al., 2016; Sindhu et al., 2022). The total antioxidant activity (Fig. 7g) of saffron is due to the cumulative effect of enzymatic and nonenzymatic pathways. Enzymatic antioxidant activity is due to the action of CAT, GR, POD, APX, and SOD (Fig. 7a, b, d, f), while nonenzymatic antioxidant activity is due to phenolics, ascorbic acid, carotenoids, and apocarotenoids. (Figs. 6a, b, 8c, e, 9a, b).

3.5. *Pigment and secondary metabolite accumulation are greater in microbially inoculated plants*

Carotenoids are the most widely distributed group of photosynthetic accessory pigments. These scavenge oxygen radicals are released from chloroplasts during photosynthesis, thus protecting cellular constituents from free radical damage. Carotenoids accumulate in the chloroplasts of all green plants as a mixture of α- and β-carotene, β-cryptoxanthin, lutein, zeaxanthin, violaxanthin, and neoxanthin. The levels of carotenoids are variable and are affected by ripeness, genotype, and cultivation methods (Abushita et al., 2000; Martínez-Valverde et al., 2002; George et al., 2004; Kuti and Konuru, 2005; Hallmann, 2012). In the present study, microbial inoculation had a significant positive effect on the total carotenoid content in saffron. In saffron, inoculation with AMF + PSB + KSB (T_3) resulted in a 32.39 % increase in total carotenoids in the saffron stigma (Fig. 8a). AMF colonization of medicinal and aromatic plants, such as *Arnica montana* L., *Coriandrum sativum* L., and *Anethum graveolens* L., influences the biosynthesis of bioactive compounds, such as ascorbic acid, flavonoids, polyphenols, carotenoids, and vitamins (Zeng et al., 2013; Giovannetti et al., 2013; Rydlová et al., 2016). Several earlier studies have shown higher levels of bioactive compounds in organically produced fruits than in conventionally produced fruits (Rembiałkowska, 2004; Chassy et al., 2006), but not all related studies are consistent in this respect. However, in one study, there was no significant difference in the carotenoid content between conventional and organic tomatoes (Caris-Veyrat et al., 2004). In another study, tomatoes produced more carotenoids under organic cultivation than under conventional cultivation (Zoran et al., 2014).

The apocarotenoids of saffron are the most important of all the saffron compounds and are responsible for its economic value. These bioactive molecules are generated from the oxidative cleavage of the double bonds of carotenoids, followed by specific glycosylation steps. In the present study, there was a positive correlation between the abundances of several microbial combinations and the contents of crocin, picrocrocin and safranal in saffron (Fig. 8b). A recent study showed that an AMF inoculum containing *R. intraradices* and *F. mosseae* was highly effective at increasing flower production and saffron yield. In contrast, *R. intraradices* alone increased the content of several bioactive compounds, such as picrocrocin, quercitrin, and crocin II, and antioxidant activity (Caser et al., 2019b). In the soilless system, *R. intraradices* inoculation increased the quality of saffron (Caser et al., 2019a).

In the present study, crocin, picrocrocin and safranal contents were significantly greater in the saffron stigma in all the treatment groups than in the control group (Fig. 8b). According to the ISO 3632 classification, the best quality saffron belongs to category I, which means that direct readings of the absorbance at approximately 440 nm, 330 nm, and 257 nm for crocin, picrocrocin, and safranal, respectively, are greater than 190, 70, and 20. Maximum crocin (325.46), picrocrocin (98.72), and safranal (55.49) were recorded on AMF + PSB + KSB (T_3), which was 64.95, 35.46, 56.16 % higher than control, respectively. These are promising results, as the values of the three apocarotenoids indicate that the saffron stigma belongs to category I. These findings confirmed that microbial inoculation produces high-quality saffron for coloring, aroma, and taste.

Many factors contribute to the differences in saffron quality. The apocarotenoid content varies with geographical location, cultivation practices, postharvest processing, and storage (Husaini et al., 2010b; Husaini et al., 2022; Manzo et al., 2015). A recent study showed that the location of the experimental site significantly affects saffron quality, but there was no direct correlation between altitude and the concentration of apocarotenoids (Sheikh et al., 2023). In another study, the total apocarotenoid content was greater in field-cultivated saffron than in indoor saffron owing to differences in gene expression (Zhou et al., 2020). Saffron corms that always remained in the field had more roots and brighter green leaves than those placed for flowering incubation inside wooden pallets without soil or water. The absorption of nutrients from the soil by roots and the ability to carry out photosynthesis enhanced apocarotenoid accumulation under field conditions. Therefore, the composition of the supporting growth medium must be important for both saffron growth and the apocarotenoid content of the stigma.

The factors that can directly or indirectly influence saffron quality include edaphic factors (the soil microbiome and mineral and organic carbon contents), weather conditions (the average temperature, rainfall, and extreme weather events), and crop management practices. At the genetic level, all of these factors can lead to the modulation of apocarotenoid gene expression, thereby influencing the content of bioactive compounds in saffron (Sheikh et al., 2023). The results of the present study indicate that microbial inoculation of saffron can be highly beneficial for overall plant growth and development and increase economic yield.

3.6. *Designing consortia of microbes for biofertilizer formulation is trait-specific and requires host-specific empirical studies*

Plant roots secrete a wide range of compounds, including amino acids (Walker et al., 2003; Weston et al., 2012; Moe, 2013), and these root exudates act as chemical attractants for several heterogeneous and highly diverse microbial communities. Therefore, a generalized recommendation for using any biofertilizer is unsuitable for a unique crop such as saffron. In the present study, AMF + PSB + KSB (T_3) was the most suitable microbial consortium for saffron, rather than *Azotobacter* + *Azospirillum* + AMF + PSB + KSB (T_4). We expected T_4 to be the best treatment because it contained the maximum number of useful microbial inoculants, especially those involved in nitrogen fixation. Our results show that it is too simplistic to presume that a greater number of microbial inoculants would lead to a better positive outcome for plant growth; therefore, before making a recommendation of BF for a crop species, one needs to validate it practically. Even though there were a maximum number of useful microbes in T_4, their anticipated positive effect could not translate into economically important traits like saffron corm production, stigma yield or quality.

The complementary intermicrobial relationships had an overall positive interactive effect (T_3) on the economically important plant traits, more than each component's simple individual additive effects (T_4). This could be due to an imbalance between endogenously and exogenously available growth hormones (IAA, NAA, cytokinin, and gibberellins) (Glick, 2012). Moreover, while designing a consortium for a biofertilizer, certain bacterial groups appear to associate more frequently with AM fungi or to be inhibited by them by several mechanisms (Filion et al., 1999; Mansfeld-Giese et al., 2002; Gupta Sood, 2003; Toljander et al., 2006; Vestergård et al., 2008; Wamberg et al., 2003; Johansson et al., 2004; Wang et al., 2021; Díez-Méndez et al., 2024). Host-specific interactions are known to regulate the structure of the soil microbial community near the root surface (Bulgarelli et al., 2013; Huang et al., 2014). The direct interactions between these different microbial types could have resulted in various interactive processes (Kour et al., 2020) that ultimately benefited the growth of the saffron plants and soil health. The present study showed that AMF significantly promoted root and shoot biomass (Fig. 4a, b) in saffron plants, especially when AMF were used in combination with PSB and KSB (T_3). Earlier studies reported that AMF influence nutrient uptake, water availability, and productivity and act as bioprotectants against pathogens and stress factors (Harrier and Watson, 2004; Akhtar and Siddiqui, 2007; Shi et al., 2023).

Azotobacter and *Azospirillum* secrete IAA, which is involved in multiple processes of cell division and vascular bundle formation. Bacterial IAA loosens plant cell walls, facilitates root exudation, and increases root length and surface area, eventually providing easier access to soil nutrients. It is a key effector molecule in plant—microbe interactions, both in pathogenesis and phytostimulation (Ahemad and Khan, 2012a, b, c). However, despite these properties of *Azotobacter* and *Azospirillum*, treatment with T_4 was not the best treatment for increasing root and shoot biomass. Although difficult to explain, endogenous IAA in plant roots can be optimal or suboptimal for growth and is vital for determining whether bacterial IAA stimulates or suppresses plant growth (Glick, 2012). Therefore, the species specificity of the strains or differences in adaptation to environmental conditions should be considered when selecting strains for the formulation of biofertilizers (Antunes et al., 2011; Zoppellari et al., 2014; Malusà et al., 2016; Shi et al., 2023).

3.7. *Choosing a trait-specific microbial consortium for a crop is the way forward*

The present study showed that beneficial microbes influence plant growth and development, change nutrient dynamics and increase tolerance by inducing systemic resistance via enzymatic and nonenzymatic mechanisms. The biofertilizer combination AMF + PSB + KSB had the greatest effect on the general growth characteristics (shoot and root growth, increased number of flowers, early flowering) and economic characteristics (maximum stigma, corm yield and big corm index) of saffron.

There was a difference in nutrient availability and uptake of soil nutrients between the biofertilizer combinations. The soil OC, N and Mo contents (at both the flowering and vegetative stages) increased significantly in the biofertilizer combination *Azotobacter* + PSB + KSB, while the P, K, Cu, Fe, Mn, and Zn contents were significantly greater in biofertilizer combination AMF + PSB + KSB. The maximum increase in the uptake of P, K, Cu, Fe, Mn, and Zn was found in AMF + PSB + KSB, while the maximum N and Mo uptake was observed in *Azotobacter* + PSB + KSB. AMF caused significant increases in elements useful for improving stress tolerance during the vegetative and flowering phases viz. P, K, Cu, Fe, Mn, and Zn.

While microbial inoculants increased nutrient uptake by saffron plants, they cause mild stress to plants, triggering biochemical defense. Biofertilizers enhanced the stress preparedness of saffron by activating enzymatic and nonenzymatic antioxidant responses. Compared with the control, the proline and glycine-betaine contents in the biofertilizer combination AMF + PSB + KSB and biofertilizer combination *Azotobacter* + *Azospirillum* + AMF + PSB + KSB significantly increased in all the plant parts. Biofertilizers caused maximum increase in PAL activity in subterranean parts, such as roots and corms, leading to induced systemic resistance (ISR). However, inoculation with AMF + PSB + KSB resulted in the highest PAL enzyme activity in the stigma, followed by that in the corm, root, and tepal. Saffron being susceptible to many soil-borne diseases, so microbial inoculation can be instrumental in enhancing stress tolerance mediated by PAL activity.

A generalized recommendation for using any biofertilizer is unsuitable for a unique crop such as saffron. AMF + PSB + KSB was the most suitable microbial consortium for saffron, rather than *Azotobacter* + *Azospirillum* + AMF + PSB + KSB. Farmers presuming that a larger number of microbial inoculants would lead to a better positive outcome for plant growth doesn't hold. The interplay of the three beneficial microbial components in the consortium (T_3) caused an overall positive interactive effect through complementation, while the cumulative effect of the 5 individual components of the microbial consortium (T_4) was lower. Therefore, before recommending biofertilizer for a crop species, one must practically validate it.

If a farmer's objective is to multiply the corms, irrespective of the size of the daughter corms, then *Azospirillum* + PSB + KSB is the most suitable microbial consortium. However, if the target is to get a large percentage of big-sized daughter corms ($\geq$ 10 g) and maximum corm yield, then AMF + PSB + KSB is the best choice. Moreover, inoculation with AMF + PSB + KSB resulted in a 32.39 % increase in total carotenoids in the saffron stigma. Maximum crocin (325.46), picrocrocin (98.72), and safranal (55.49) were recorded on AMF + PSB + KSB, which was 64.95, 35.46, 56.16 % higher than control, respectively. The values of the three apocarotenoids indicated that the saffron stigma belonged to category I and that the trait-specific organic production module produces high-quality saffron for coloring, aroma, and taste (Fig. 9 a,b).

So, the choice of biofertilizers determines the nature of qualitative and quantitative improvement in a crop's agronomically important target traits. Improving soil and plant health by identifying external microbial inoculations and shifting the host-specific symbiotic soil microbiome equilibrium are viable strategies for sustainable saffron production (Khan et al., 2023).

4. Conclusion

Saffron is not easy to grow, especially as many biotic and abiotic stresses adversely affect its productivity and quality. The biofertilizer application increased the counts of effective microbes in the saffron rhizosphere, depicting that the native microbial population had no significant inhibitory effect on the microbial inoculants, and there was no antagonism between the inoculants. Significant interactions existed between *Crocus sativus* and the microbial inoculants, which differed qualitatively and quantitatively. The present study discovered an efficient Crocus growth-promoting microbial consortium for saffron farmers' use and commercialization. The best consortia for saffron constitutes of *Bacillus megaterium*, *Bacillus subtilis strain DR2*, *Pseudomonas putida*, *Mesorrhizobium* sp. and AMF, which predominantly contained *Glomus.* This biofertilizer combination had the greatest positive effect on the general growth characteristics (shoot and root growth, increased number of flowers, early flowering) and economic characteristics (maximum stigma yield, corm yield, stigma quality and big corm index) of saffron, with AMF having a major positive impact (comparing T_3 and T_2). Enhancing stigma quality and antioxidant potential via the consortium can add to its market value as a medicinally important cash crop. While designing a consortium for a biofertilizer for a crop species, certain bacterial groups may appear to associate better with AM fungi and each other. So, choosing the right biofertilizer combination determines its qualitative and quantitative effect on a crop's agronomic trait. There is a need to develop crop-specific microbial consortia for other crops and elucidate the underlying mechanisms of those organic-production modules.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

A.M.H. conceptualized, designed and provided facilities for the research; S.N., N.B and A.M.H. performed the research; AMH, FAN and

SAM performed the analysis; and A.M.H. wrote the article with input from all the authors. All the authors contributed to the article and approved the submitted version.

Funding

The corresponding author is thankful to the National Mission on Himalayan Studies, Ministry of Environment, Forests and Climate change, New Delhi, India for the financial assistance provided for saffron research.

CRediT authorship contribution statement

Neha Bharti: Writing – original draft, Validation, Investigation, Data curation. **Amjad M. Husaini:** Writing – review & editing, Supervision, Software, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Firdous A. Nehvi:** Writing – review & editing, Supervision, Methodology. **Shakeel A. Mir:** Writing – review & editing, Methodology, Formal analysis. **Samiullah Naik:** Writing – original draft, Investigation, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

No AI-Based tools were used to write the paper

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amjad M. Husaini reports financial support was provided by National Mission on Himalayan Studies, Ministry of Environment, Forests and Climate Change, India. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors are thankful to the National Mission on Himalayan Studies for the financial assistance provided for saffron research. The authors are highly grateful to Mr. Anam ul Haq for helping with the figures, and the SKUAST-K administration for the support and facilities.

Informed consent

Not applicable

Code availability

Not applicable

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Article *in* Journal of Crop Science and Biotechnology · September 2024

DOI: 10.1007/s12892-024-00260-5

Deepika Pandita

Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Immad A Shah

Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir

Abdel Rahman Mohammad Al Tawaha

Al-Hussein Bin Talal University

Journal of Crop Science and Biotechnology
https://doi.org/10.1007/s12892-024-00260-5

ORIGINAL RESEARCH

Nutritional profiles and fertilizer responsiveness of cold-adapted rice germplasm of Kashmir Himalayan region: creating a high-value germplasm repository for smart-breeding biofortified rice

Deepika Pandita[1] · Immad A. Shah[2] · Mudasir A. Mir[3] · Najeebul Rehman Sofi[4] · Abdel Rahman Al Tawaha[5] · Amjad M. Husaini[1]

Accepted: 2 September 2024

Abstract

Rice (*Oryza sativa* L.), a pivotal cereal crop globally, plays a crucial role in addressing nutritional deficiencies affecting half of the world's 8.1 billion population. Developed nations combat these deficiencies through fortification programs, yet such initiatives remain economically unfeasible for developing countries. Hence, cost-effective strategies are imperative. In this study, 50 rice genotypes sourced from the Himalayan germplasm collection were meticulously evaluated for their nutritional profiles encompassing 14 key minerals and nutrients: phosphorus, iron, potassium, magnesium, calcium, sodium, zinc, protein, starch, amylose, amylopectin, and crude fiber. Furthermore, these genotypes underwent assessment for fertilizer responsiveness following recommended dosages and schedules. Pre- and post-treatment nutritional analyses were conducted to ascertain genetic potential in mineral uptake and to explore inter-correlations among these nutrients. Most fertilizer-responsive genotypes were clustered together, indicating similar response patterns. Complex correlations were observed within these germplasm groups, notably a significant positive correlation between calcium and sodium, and a negative correlation between phosphorus and potassium. Moreover, a positive correlation was found between protein and crude fiber, while protein exhibited a negative correlation with amylopectin. The identification of several high-nutritive-value genotypes, demonstrating enhanced cumulative mineral uptake or nutrient content, underscores their potential utility in breeding programs aimed at developing biofortified, cold-tolerant rice varieties tailored for the North-western Himalayan region of Kashmir valley in India.

Keywords Rice · Genetic diversity · Nutritional value · Uptake · Himalayas · Breeding

Introduction

Rice (*Oryza sativa* L.) is a dietary staple food and one of the most important cereal crops, especially for people in Asia (Orthoefer and Eastman 2005). In order to safeguard the livelihood security of rice farmers and the nutritional security of the population, it is important to conduct research oriented towards increasing farmers' income. Consumer acceptability and their willingness to pay more would depend on the nutritional quality of food. Biofortification of industrial products is usually carried out to cater to the demand of such high-income consumers, but such products are not affordable to the low-income common man due to their high price. Biofortification can be done either through the generation of biofortified lines using genetic approach or by externally adding micronutrients/ minerals

✉ Amjad M. Husaini
amjadhusaini@skuastkashmir.ac.in

[1] Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, SKUAST-K, Shalimar, Srinagar, Jammu and Kashmir 190025, India

[2] Division of Agricultural Statistics, SKUAST-K, Srinagar, India

[3] Division of Plant Biotechnology, SKUAST-K, Srinagar, India

[4] MRCFC, Khudwani, SKUAST-K, Srinagar, India

[5] Department of Biological Sciences, Al Hussein Bil Talal University, Maan, Jordan

during postharvest packaging practices; however the genetic approach is more sustainable and affordable.

Kashmir is known for temperate rice, grown in valley plains (1500–1650 m amsl) and cold-tolerant rice, grown in high-altitude areas 1800–2400 m amsl). These rice genotypes are different from the typical sub-tropical/tropical rice genotypes in the rest of India, especially concerning cold tolerance (Husaini et al. 2021; Husaini and Sofi 2018). In addition, the Himalayan region of Kashmir produces some 'specialty' rice varieties which have traditionally been fed to pregnant women due to their high nutritional quality and numerous health-associated benefits (increasing antioxidant potential) (Husaini et al. 2013). Despite this, most rice breeding programmes in the region have mainly overlooked nutritional quality and consumer acceptability in favour of improving disease resistance, cold tolerance, or high yields (Sanghera et al. 2010, 2011). The biological diversity of indigenous rice has been eroded as a result of crop diversification and land diversion towards more remunerative horticultural crops and high-yielding varieties. Furthermore, physiological imbalance in mineral uptake, and environmental stresses (low temperature and water scarcity) coupled with cultivation under marginal conditions have harmed the qualitative and quantitative properties of rice germplasm.

Consumers who are concerned about their health are interested in having rice with good nutritional value. Knowledge about the nutritional status of rice is becoming increasingly popular among consumers because of nutritional deficiency disorders. Low micronutrient density in staple foods is generally the primary reason for human micronutrient malnutrition in developing countries (Cakmak 2008, 2009; Gibson 2006; Husaini and Sohail 2023; Welch and Graham 2004). Optimal growth needs a correct diet in terms of adequate intake of macronutrients and micronutrients. Macronutrients are the compounds that humans consume in largest quantities, mainly classified in carbohydrates, proteins and fats. Micronutrients are instead introduced in small quantities, but they are required for an adequate growth in the pediatric age, especially zinc, iron, vitamin D and folic acid (Brown et al. 2001; Miller 2013; Prasad 2013; Roohani et al. 2013; Savarino et al. 2021). Biofortification of micronutrient contents in staple foods through agronomic or genetic approaches has attracted increasing interest during the past decades (Husaini 2022; Wani and Husaini 2021). Hence, the identification and revival of 'indigenous high-value' rice genotypes have assumed special importance under the present scenario.

The nutritional makeup of the various rice types available in India needs to be evaluated, especially those of the temperate rice of Himalayan region in northern India (Ashraf et al. 2017). In a previous study, we analyzed the same set of 50 rice genotypes using the Neighbour-Joining clustering method and found that 20 were grouped in cluster I and 15 each grouped in clusters II and III (Pandita et al. 2023). In continuation of the analysis of rice genotypes using the neighbour-joining method by (Pandita et al. 2023), this study was aimed to estimate the mineral composition and nutritional quality of the Himalayan rice germplasm set comprising of high altitude genotypes, valley temperate genotypes and basmati genotypes adapted to Kashmir valley. Furthermore the study aimed to know the correlation between the genetic diversity groups and their nutritional profile by evaluating the response of these genotypes to fertilizer application.

Materials and methods

Plant material

A total of 50 rice genotypes collected from different regions of North West Himalayas and some promising lines that were maintained at the Mountain Research Centre for Field Crops (MRCFC), Khudwani, Anantnag, J&K were used for the study. The selected rice accessions used were i) High altitude genotypes grown between 1800–2100 amsl (HA-1, HA-2, HA-3, HA-4, HA-5, HA-6, HA-7, HA-8, HA-10, HA-11); ii) Valley temperate genotypes cultivated between 1650–1800 amsl (K-12, K-13, K-14, K-15, K-16, K-17, K-19, K-20, K-21, K-22, K-23, K-24, K-25, K-27, K-28, K-29, K-30, K-31, K-32, K-33, K-34, K-35, K-36, K-37, K-38, K-39, K-40, K-41, K-42, K-43, K-44, K-45, K-47, K-48, K-50, K-51, K-52), iii) and Basmati type genotypes (B-53, B-54, B-55) (Pandita et al. 2023). The seeds of these rice accessions were germinated in seed germinator at 30 ± 2 ℃ in the petriplates. After 5 days the germinated seeds were transferred into portrays containing clay soil and incubated in walkin plant growth chamber at 25 ± 2 ℃. Thirty day old healthy seedlings (about 15 cm tall) were transplanted in field in two adjacent plots @2–3 seedlings per hill with spacing of 15 × 15 cm. Before transplanting, one plot was fertigated (treated) with fertilizer combination containing the recommended dosage of phosphorous @70 kg/ha, iron @15 kg/ha, potassium @20 kg/ha, magnesium @40 kg/ha, calcium @200 kg/ha, sodium @20 kg/ha and zinc @15 kg/ha (Kumar et al. 2017), (Aslam et al. 2001), while the other plot (control) was left without any external nutrient application. The package of practices recommended by SKUAST-K, India were followed till the harvest. After harvesting and threshing, all the grains of 50 different rice varieties were collected from both plots and were stored (Fig. 1). The grain samples were de-husked and grinded manually using pestle mortar. The grinded samples were used to estimate minerals and nutrients.

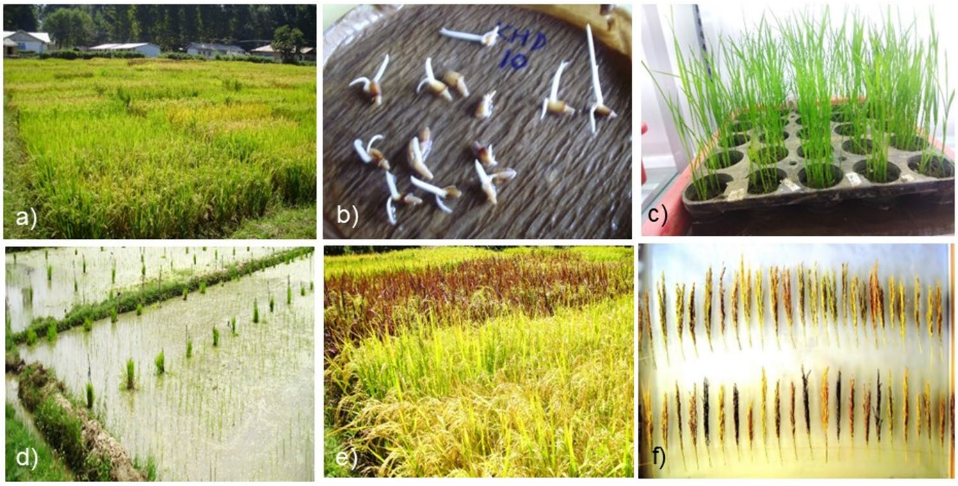

Fig. 1 The fifty rice genotypes of Kashmir Himalayas; **a** Crop cafeteria at maturity (1st year); **b** Seed germination of each genotype in petriplates; **c** Seeding development in pro-trays; **d** Transplantation in field; **e** Variability among some genotypes at maturity (2nd year); **f** Panicle variabilitiy after harvest

Estimation of mineral and nutrient contents

For mineral estimation, 0.5 g of powdered rice sample was taken in a flask for each mineral, and then 20 ml of di-acid mixture of nitric acid and perchloric acid in a ratio of 9:4 was added. The flask was kept undisturbed overnight at room temperature (25 ± 2 °C) and, on the next day, placed on a hot plate at 115–118 °C for digestion (approx. for 3 h) till the solution became transparent. The digested samples were then filtered with the 0.22 µm size Whatman filter paper. To ensure a complete transfer of digested material, about six washings were given with double distilled water and the final volume was made to 100 ml, which was ultimately used to estimate the minerals.

Magnesium, iron, and zinc were estimated on an atomic absorption spectrometer (Model: FS 224, Make: Agilent Technologies and Mode: Flame mode) using standard protocol (Filgueiras et al. 2000). Calcium, potassium, and sodium were determined through flame excitation using a flame photometer (Hald and Mason 1958). Phosphorous was determined using a double beam spectrometer (Agilent Technologies, Model: Carry-100) (Derikvandi and Nezamzadeh-Ejhieh 2017).

Half a gram of de-husked rice samples in powdered form per replicate was used to determine nitrogen content using micro-Kjeldahl method (Kjeldahl 1883). The content of starch (Thayumanavan and Sadasivam 1984), total amylose (Sadasivam and Manickam 1992), Amylopectin content (Chattopadhyay et al. 2008) and crude fibre (Maynard 1970) were determined by using standard methods.

Statistical analysis

In a comparative analysis of 50 rice genotypes across treated and untreated plots, ANOVA was employed at a chosen significance level of 5% (Kim 2014). Post hoc comparisons were conducted using the Least Significant Difference method (Williams and Abdi 2010). Descriptive statistics, expressed as Mean ± Standard Error, were utilized to characterize genotype performance for the measured parameters. The uptake percentage of minerals was determined. Similarly, the nutritional profile of genotypes, also expressed as Mean ± SE, was analyzed, and the percent increase in nutritional profile concerning both control and treated plots was calculated. For graphical representations, box-plots were generated along with corresponding *p*-values using the ggplot package (Wickham et al. 2016). Additionally, a correlation matrix was computed for the studied parameters, with corresponding *p*-values for the correlation coefficients. Data analysis and visualization were performed using R Studio version 4.2.2.

Results

Phosphorous (P)

The highest level of P concentration was found in genotype (control) K-37 (4553.33 ppm), and the lowest level was found in genotype (control) K-44 (2133.33 ppm) (Table 1, Fig. 13S). In terms of P uptake, B-55 (treated) had the highest level of P uptake among the 50 genotypes, with 21.4% increased levels from 2476.67 to 3006.67 ppm, while K-39 (treated) had the lowest level of P uptake with 0.62% increased levels only (Table 1, Fig. 1S). The *p*-value of the test for P is 0.0001, which is lower than the significance level alpha = 0.05. The results infer that the effect of treatment on P uptake among genotypes was significant post-treatment. The uptake of mineral P by rice genotypes was statistically significant. The box plot and the corresponding *p*-value are shown in Fig. 2a.

Table 1 Mineral profile of different genotypes and uptake of Phosphorus, Iron, Potassium and Zinc in response to fertilizer application

S. No	Genotype	Phosphorus (ppm)			Iron (ppm)			Potassium (ppm)			Zinc (ppm)		
		(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %
		Control	Treated		Control	Treated		Control	Treated		Control	Treated	
1	HA-1	3766.67 ± 17.64	3796.67 ± 20.28	0.80	6.93 ± 0.35	8.13 ± 0.35	17.32	1359.33 ± 5.21	1380.67 ± 2.91	1.57	3.64 ± 0.04	4.24 ± 0.04	16.48
2	HA-2	4206.67 ± 26.03	4443.33 ± 20.28	5.63	17.4 ± 0.42	19.87 ± 0.29	14.20	1622 ± 4.16	2081.33 ± 4.67	28.32	2.13 ± 0.03	2.27 ± 0.04	6.57
3	HA-3	3913.33 ± 26.03	4186.67 ± 20.28	6.98	14.33 ± 0.47	14.73 ± 0.47	2.79	1061.33 ± 3.53	1061.33 ± 3.53	0.00	4.33 ± 0.05	5.87 ± 0.04	35.57
4	HA-4	3983.33 ± 26.03	4510 ± 20.82	13.22	24.33 ± 0.47	30.13 ± 0.35	23.84	1100.67 ± 4.06	1380.67 ± 5.21	25.44	3.67 ± 0.04	4.25 ± 0.04	15.80
5	HA-5	4036.67 ± 23.33	4116.67 ± 14.53	1.98	20.67 ± 0.29	21.73 ± 0.52	5.13	1100.67 ± 4.06	1180.67 ± 4.06	7.27	3.71 ± 0.05	4.23 ± 0.05	14.02
6	HA-6	3503.33 ± 20.28	3756.67 ± 26.03	7.23	15.13 ± 0.29	16.73 ± 0.35	10.58	1281.33 ± 4.67	1381.33 ± 5.81	7.80	1.99 ± 0.03	3.63 ± 0.04	82.41
7	HA-7	3786.67 ± 20.28	4006.67 ± 23.33	5.81	24.13 ± 0.47	27.93 ± 0.41	15.75	1380.67 ± 4.06	1420.67 ± 5.21	2.90	1.75 ± 0.03	2.01 ± 0.04	14.86
8	HA-8	3203.33 ± 17.64	3556.67 ± 12.02	11.03	15.93 ± 0.24	22.53 ± 0.47	41.43	1639.33 ± 4.06	1681.33 ± 5.81	2.56	4.93 ± 0.05	7.21 ± 0.04	46.25
9	HA-10	3023.33 ± 14.53	3303.33 ± 20.28	9.26	13.53 ± 0.47	18.73 ± 0.47	38.43	1821.33 ± 5.81	1881.33 ± 3.53	3.29	2.47 ± 0.04	2.59 ± 0.04	4.86
10	HA-11	3006.67 ± 23.33	3496.67 ± 26.03	16.30	18.6 ± 0.42	19.87 ± 0.29	6.83	1420.67 ± 4.06	1518 ± 4.16	6.85	6.59 ± 0.06	7.11 ± 0.04	7.89
11	K-12	3446.67 ± 23.33	3716.67 ± 17.64	7.83	13.87 ± 0.41	17.73 ± 0.47	27.83	1621.33 ± 5.81	1841.33 ± 4.67	13.57	2.35 ± 0.05	4.27 ± 0.05	81.70
12	K-13	4123.33 ± 26.03	4510 ± 20.82	9.38	31.93 ± 0.41	36.13 ± 0.47	13.15	1300.67 ± 4.06	1380.67 ± 4.06	6.15	6.05 ± 0.04	6.19 ± 0.04	2.31
13	K-14	4326.67 ± 26.03	4563.33 ± 20.28	5.47	5.93 ± 0.35	8.73 ± 0.82	47.22	1099.33 ± 5.21	1160.67 ± 2.91	5.58	2.17 ± 0.05	3.65 ± 0.04	68.20
14	K-15	4106.67 ± 17.64	4253.33 ± 20.28	3.57	17.8 ± 0.42	22.07 ± 0.41	23.99	1219.33 ± 2.91	1241.33 ± 3.53	1.80	1.03 ± 0.05	2.03 ± 0.05	97.09
15	K-16	3126.67 ± 20.28	3510 ± 20.82	12.26	12.93 ± 0.35	19.53 ± 0.35	51.04	1059.33 ± 2.91	1319.33 ± 2.91	24.54	2.11 ± 0.05	2.25 ± 0.04	6.64
16	K-17	3440 ± 20.82	3796.67 ± 23.33	10.37	3.47 ± 0.29	6.73 ± 0.47	93.95	921.33 ± 4.67	999.33 ± 2.91	8.47	6.13 ± 0.04	6.17 ± 0.05	0.65
17	K-19	4193.33 ± 20.28	4326.67 ± 26.03	3.18	21.73 ± 0.41	41.33 ± 0.24	90.20	921.33 ± 6.96	1261.33 ± 5.81	36.90	2.17 ± 0.03	2.21 ± 0.05	1.84
18	K-20	4233.33 ± 20.28	4396.67 ± 17.64	3.86	10.53 ± 0.52	13.73 ± 0.41	30.39	1279.33 ± 4.06	1378.67 ± 4.67	7.77	6.17 ± 0.05	6.25 ± 0.04	1.30
19	K-21	4446.67 ± 23.33	4566.67 ± 20.28	2.70	2.27 ± 0.41	3.53 ± 0.87	55.51	1059.33 ± 4.06	1141.33 ± 3.53	7.74	4.73 ± 0.04	4.85 ± 0.04	2.54
20	K-22	4213.33 ± 14.53	4276.67 ± 17.64	1.50	15.13 ± 0.24	17.87 ± 0.41	18.11	1219.33 ± 2.91	1318.67 ± 3.53	8.15	4.63 ± 0.03	4.91 ± 0.04	6.05
21	K-23	4103.33 ± 14.53	4426.67 ± 17.64	7.88	12.73 ± 0.24	15.47 ± 0.41	21.52	1239.33 ± 4.06	1361.33 ± 5.81	9.84	4.91 ± 0.04	5.97 ± 0.04	21.59
22	K-24	4223.33 ± 14.53	4256.67 ± 23.33	0.79	11.93 ± 0.52	14.27 ± 0.35	19.61	1101.33 ± 3.53	1181.33 ± 5.81	7.26	6.27 ± 0.05	7.51 ± 0.05	19.78
23	K-25	4320 ± 20.82	4446.67 ± 23.33	2.93	13.87 ± 0.52	16.53 ± 0.41	19.18	1239.33 ± 4.06	1299.33 ± 5.21	4.84	2.23 ± 0.06	4.35 ± 0.04	95.07
24	K-27	4093.33 ± 23.33	4343.33 ± 20.28	6.11	15.33 ± 0.35	17.73 ± 0.41	15.66	1142 ± 4.16	1418.67 ± 3.53	24.23	4.53 ± 0.04	5.17 ± 0.04	14.13
25	K-28	3206.67 ± 20.28	3263.33 ± 14.53	1.77	11.13 ± 0.58	17.67 ± 0.52	58.76	1320.67 ± 2.91	1418.67 ± 3.53	7.42	7.17 ± 0.05	7.45 ± 0.04	3.91
26	K-29	3296.67 ± 17.64	3356.67 ± 23.33	1.82	5.13 ± 0.35	5.67 ± 0.41	10.53	998 ± 4.16	1061.33 ± 5.81	6.35	1.53 ± 0.04	1.57 ± 0.04	2.61
27	K-30	2233.33 ± 14.53	2446.67 ± 17.64	9.55	14.73 ± 0.35	22.53 ± 0.41	52.95	1401.33 ± 4.67	1401.33 ± 4.67	0.00	2.03 ± 0.05	3.61 ± 0.04	77.83
28	K-31	2506.67 ± 17.64	2746.67 ± 17.64	9.57	3.13 ± 0.58	5.67 ± 0.24	81.15	1361.33 ± 3.53	1481.33 ± 5.81	8.81	6.75 ± 0.04	7.35 ± 0.04	8.89
29	K-32	3093.33 ± 17.64	3336.67 ± 23.33	7.87	17.07 ± 0.41	17.93 ± 0.47	5.04	1841.33 ± 6.96	1879.33 ± 4.06	2.06	4.53 ± 0.04	5.13 ± 0.04	13.25
30	K-33	2593.33 ± 14.53	3006.67 ± 17.64	15.94	11.27 ± 0.41	15.93 ± 0.47	41.35	1241.33 ± 4.67	1341.33 ± 6.96	8.06	6.27 ± 0.04	6.49 ± 0.04	3.51
31	K-34	3320 ± 20.82	3446.67 ± 17.64	3.82	5.53 ± 0.47	8.73 ± 0.93	57.87	1101.33 ± 6.96	1780.67 ± 4.06	61.68	10.17 ± 0.04	10.35 ± 0.04	1.77
32	K-35	2816.67 ± 17.64	2970 ± 20.82	5.44	11.93 ± 0.47	14.53 ± 0.52	21.79	1319.33 ± 4.06	1360.67 ± 4.06	3.13	9.31 ± 0.04	9.65 ± 0.04	3.65
33	K-36	2753.33 ± 23.33	2816.67 ± 17.64	2.30	17.87 ± 0.52	19.53 ± 0.47	9.29	1379.33 ± 2.91	1621.33 ± 4.67	17.54	7.89 ± 0.04	7.91 ± 0.03	0.25

Table 1 (continued)

S. No	Genotype	Phosphorus (ppm)			Iron (ppm)			Potassium (ppm)			Zinc (ppm)		
		(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %
		Control	Treated		Control	Treated		Control	Treated		Control	Treated	
34	K-37	4553.33 ± 23.33	4666.67 ± 29.06	2.49	14.27 ± 0.52	16.73 ± 0.47	17.24	1139.33 ± 2.91	1218 ± 4.16	6.90	2.53 ± 0.05	2.53 ± 0.04	0.00
35	K-38	4176.67 ± 20.28	4213.33 ± 20.28	0.88	13.53 ± 0.29	17.13 ± 0.41	26.61	1639.33 ± 4.06	1681.33 ± 5.81	2.56	7.73 ± 0.05	7.99 ± 0.03	3.36
36	K-39	4320 ± 20.82	4346.67 ± 23.33	0.62	25.87 ± 0.35	28.13 ± 0.35	8.74	1381.33 ± 5.81	1679.33 ± 4.06	21.57	3.35 ± 0.05	3.89 ± 0.05	16.12
37	K-40	3413.33 ± 26.03	3916.67 ± 17.64	14.75	10.2 ± 0.42	12.13 ± 0.35	18.92	1161.33 ± 4.67	1161.33 ± 4.67	0.00	7.03 ± 0.03	7.22 ± 0.04	2.70
38	K-41	2996.67 ± 20.28	3223.33 ± 20.28	7.56	6.73 ± 0.29	8.8 ± 0.42	30.76	1620.67 ± 4.06	1741.33 ± 5.81	7.45	6.05 ± 0.05	6.19 ± 0.05	2.31
39	K-42	3330 ± 20.82	3366.67 ± 20.28	1.10	13.73 ± 0.29	19.53 ± 0.41	42.24	1501.33 ± 3.53	1582 ± 4.16	5.37	3.45 ± 0.04	4.25 ± 0.05	23.19
40	K-43	2286.67 ± 14.53	2766.67 ± 17.64	20.99	11.93 ± 0.47	16.13 ± 0.29	35.21	1281.33 ± 3.53	1338.67 ± 3.53	4.48	1.27 ± 0.05	1.33 ± 0.04	4.72
41	K-44	2133.33 ± 23.33	2243.33 ± 23.33	5.16	9.33 ± 0.41	15.87 ± 0.29	70.10	1158 ± 4.16	1540.67 ± 4.06	33.05	4.37 ± 0.04	4.81 ± 0.04	10.07
42	K-45	3496.67 ± 20.28	3596.67 ± 17.64	2.86	9.73 ± 0.24	12.53 ± 0.29	28.78	1179.33 ± 4.06	1261.33 ± 3.53	6.95	3.05 ± 0.04	5.29 ± 0.03	73.44
43	K-47	4006.67 ± 29.06	4290 ± 20.82	7.07	14.27 ± 0.41	16.87 ± 0.29	18.22	1321.33 ± 5.81	1381.33 ± 4.67	4.54	2.37 ± 0.05	2.79 ± 0.04	17.72
44	K-48	2506.67 ± 23.33	2626.67 ± 26.03	4.79	17.47 ± 0.41	19.47 ± 0.52	11.45	1558.67 ± 3.53	1641.33 ± 4.67	5.30	5.13 ± 0.04	5.95 ± 0.04	15.98
45	K-50	4436.67 ± 26.03	4566.67 ± 17.64	2.93	14.13 ± 0.41	14.73 ± 0.35	4.25	1361.33 ± 6.96	1380.67 ± 2.91	1.42	2.87 ± 0.04	3.15 ± 0.04	9.76
46	K-51	4206.67 ± 23.33	4296.67 ± 20.28	2.14	9.33 ± 0.35	12.53 ± 0.41	34.30	1240.67 ± 4.06	1240.67 ± 2.91	0.00	4.33 ± 0.05	5.85 ± 0.04	35.10
47	K-52	3223.33 ± 14.53	3380 ± 20.82	4.86	8.07 ± 0.41	12.13 ± 0.47	50.31	1341.33 ± 5.81	1360.67 ± 5.21	1.44	2.53 ± 0.04	2.63 ± 0.04	3.95
48	B-53	2176.67 ± 23.33	2306.67 ± 17.64	5.97	14.87 ± 0.41	15.73 ± 0.29	5.78	1159.33 ± 4.06	1459.33 ± 4.06	25.88	6.51 ± 0.05	7.95 ± 0.04	22.12
49	B-54	4206.67 ± 14.53	4316.67 ± 20.28	2.61	16.93 ± 0.35	19.4 ± 0.42	14.59	1378.67 ± 4.67	1661.33 ± 3.53	20.50	4.75 ± 0.05	5.05 ± 0.05	6.32
50	B-55	2476.67 ± 17.64	3006.67 ± 17.64	21.40	20.87 ± 0.41	21.67 ± 0.47	3.83	1921.33 ± 3.53	1998 ± 4.16	3.99	6.95 ± 0.05	7.69 ± 0.05	10.65
	Mean	3531.87	3741.67		13.79	17.19		1310.37	1438.88		4.41	5.07	
	C.D	13.651	12.489		0.282	0.246		3.44	3.334		0.028	0.021	
	SE(d)	6.868	6.284		0.142	0.124		1.732	1.677		0.014	0.011	
	SE(m)	4.857	4.443		0.100	0.087		1.225	1.186		0.010	0.008	
	CV	0.238	0.206		1.262	0.861		0.162	0.143		0.393	0.257	

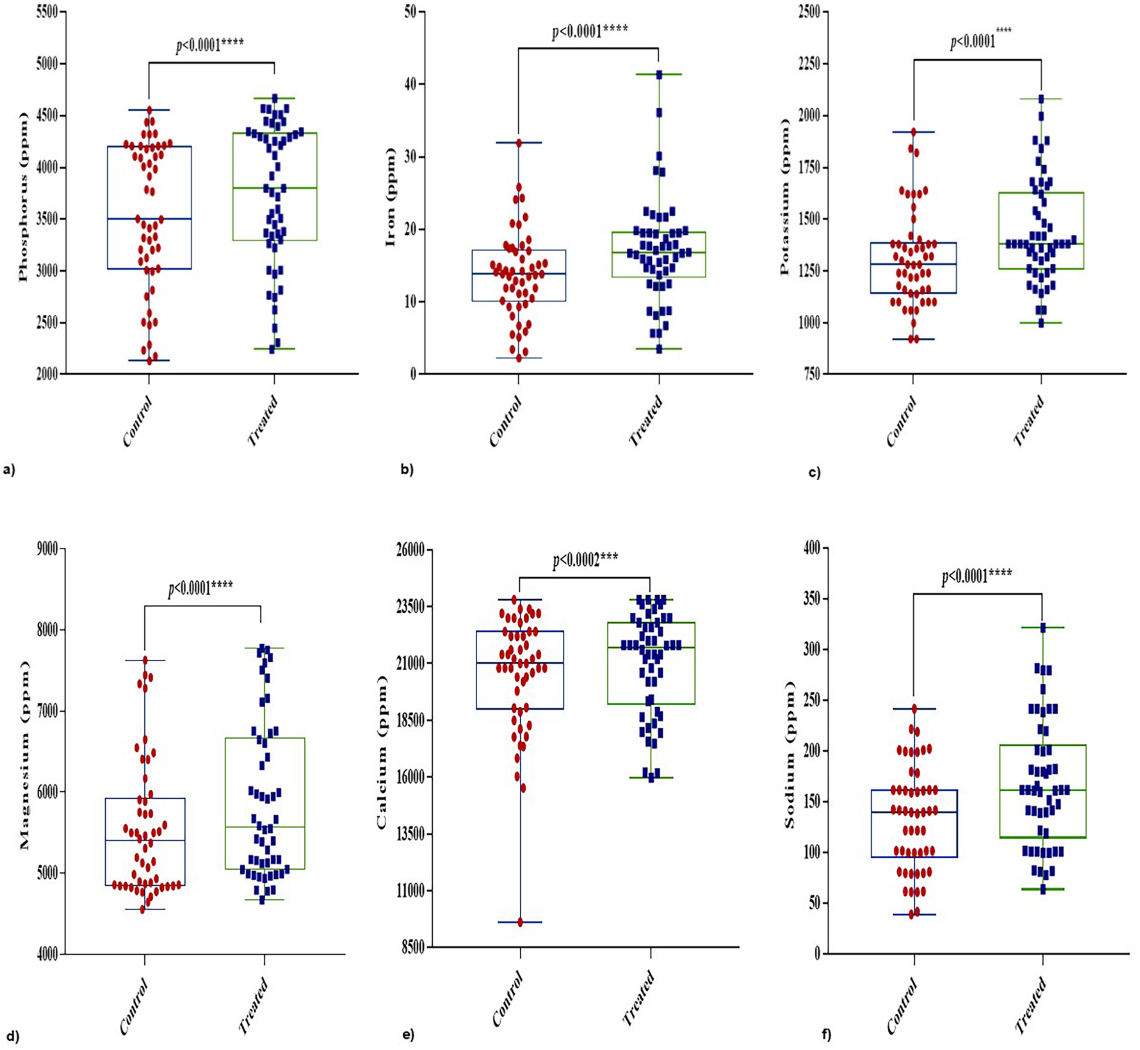

Fig. 2 Box–Whisker Plot along with *p*-value for comparison between the treated and control group **a** Phosphorus; **b** Iron; **c** Potassium; **d** Magnesium; **e** Calcium; **f** Sodium; **g** Zinc; **h** Protein; **i** Starch; **j** Amylose; **k** Amylopectin; **l** Fibre

Iron (Fe)

The highest level of iron concentration was found in genotype (control) K-13 (31.93 ppm), and the lowest level was observed in genotype (control) K-21 (2.27 ppm) (Table 1, Fig. 14S). The genotype K-17 (treated) had the highest statistically significant level of uptake among all 50 genotypes (93.95%), ranging from 3.47 to 6.73 ppm. Whereas the genotype HA-3 (treated) had the lowest statistically significant level of uptake (2.79%) only (Table 1, Fig. 2S). Fe has a *p*-value of 0.0001, which is lower than the significance level of alpha = 0.05. With a *p*-value of 0.0001 for the mineral Fe, we may conclude that the overall effect of treatment was significant among the genotypes. As a result, the data showed that the mineral Fe uptake for the rice genotypes was statistically significant. The box plot and the corresponding p-value is shown in Fig. 2b.

Potassium (K)

The highest level of K concentration was found in genotype (control) B-55 (1921.33 ppm), while a lower level was found in genotype (control) K-17 (921.33 ppm) (Table 1, Fig. 15S). Regarding uptake levels, K-34 (treated) showed the highest K uptake among the 50 genotypes. The concentration of K got elevated from 1101.33 ppm to 1780.66 ppm, which is a 61.6% increase. The genotypes HA-3 (1061.33 ppm), K-30 (1401.33 ppm), K-40 (1161.33 ppm) and K-51 (1240.67 ppm) had no significant change in the K concentration after treatment (Table 1, Fig. 3S). The *p*-value of the test for K is 0.0001, which is less than the significance level alpha = 0.05. We can conclude that the treatment effect was significant concerning K uptake among the genotypes. The mineral K uptake for

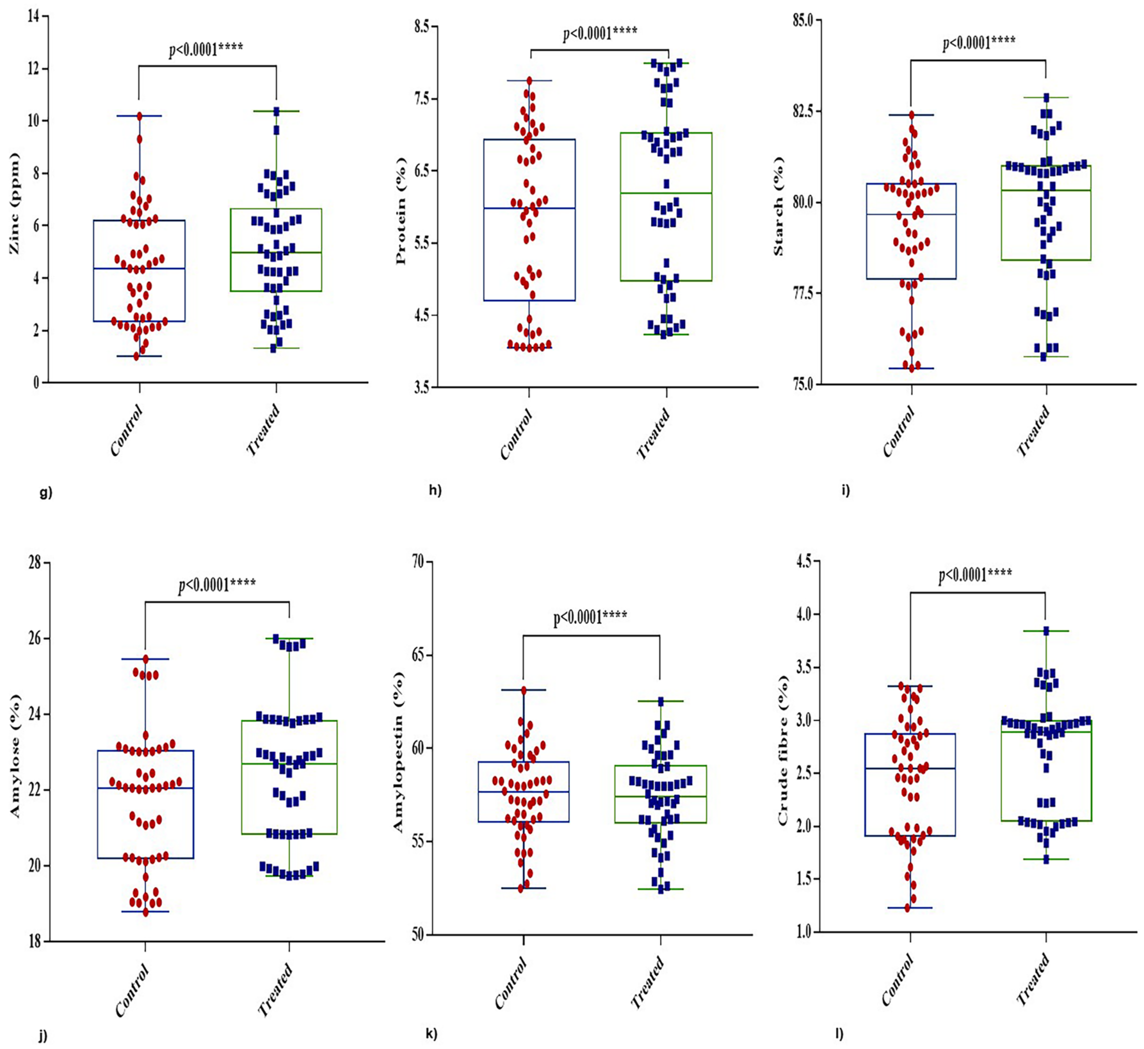

Fig. 2 (continued)

the rice genotypes was statistically significant. The box plot and the corresponding *p*-value is shown in Fig. 2c.

Magnesium (Mg)

Magnesium concentration was highest in genotype (control) K-35 (7625.33 ppm) and lowest in genotype (control) K-16 (4551.33 ppm) (Table 2, Fig. 16S). The genotype K-40 (treated) exhibited the highest statistically significant uptake (48.56%), ranging from 4984.67 to 7405.33 ppm, on the other hand the genotypes HA-3 & K-21 (treated) exhibited the lowest statistically significant level of uptake (0.27%) (Table 2, Fig. 4S). The test for Mg has a *p*-value of 0.0001, which is lower than the significance level of alpha = 0.05. The findings suggested that the effect of treatment on Mg uptake among genotypes was significant post-treatment. The box plot along with the corresponding *p* value is shown in Fig. 2d.

Calcium (Ca)

The highest level of calcium was found in genotype (control) K-13 (23,799.33 ppm), and the lowest level in genotype (control) K-19 (9618 ppm) (Table 2, Fig. 17S). K-19 (treated) had the highest statistically significant level of uptake among all 50 genotypes (66.1%) ranging from 9618 to 15,980.67 ppm. There was no significant change in the genotypes K-13 (23,799.33 ppm), K-14 (23,001.33 ppm), K-16 (23,199.33 ppm) and K-24 (18,881.33 ppm) with respect to Ca concentration after the treatment (Table 2, Fig. 5S). Ca has a *p*-value of 0.0002, which is lower than the significance level of alpha = 0.05. Regarding Ca uptake, we may conclude that the overall effect of treatment was significant among the genotypes. The mineral Ca uptake for the rice genotypes was statistically significant. The box plot and the corresponding p-value is shown in Fig. 2e.

Table 2 Mineral profile of different genotypes and uptake of Magnesium, Calcium and Sodium in response to fertilizer application

S. No	Genotype	Magnesium (ppm)			Calcium (ppm)			Sodium (ppm)		
		(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %
		Control	Treated		Control	Treated		Control	Treated	
1	HA-1	5125.33 ± 3.53	5155.33 ± 4.06	0.59	22,400.67 ± 2.91	23,001.33 ± 3.53	2.68	160.67 ± 2.91	279.33 ± 3.53	73.85
2	HA-2	5551.33 ± 4.06	5673.33 ± 3.53	2.20	21,398.67 ± 4.67	21,798 ± 4.16	1.87	160.67 ± 4.06	181.33 ± 4.67	12.86
3	HA-3	4764.67 ± 5.21	4777.33 ± 5.81	0.27	20,401.33 ± 3.53	20,599.33 ± 4.06	0.97	200.67 ± 4.06	238 ± 4.16	18.60
4	HA-4	6402.67 ± 4.67	6601.33 ± 4.06	3.10	15,521.33 ± 2.4	16,181.33 ± 3.53	4.25	60.67 ± 2.91	81.33 ± 3.53	34.05
5	HA-5	6548 ± 4.16	6728.67 ± 4.67	2.76	18,121.33 ± 4.67	18,358.67 ± 3.53	1.31	138 ± 4.16	179.33 ± 5.21	29.95
6	HA-6	5887.33 ± 4.67	6325.33 ± 4.67	7.44	22,999.33 ± 5.21	23,599.33 ± 5.21	2.61	221.33 ± 5.81	241.33 ± 3.53	9.04
7	HA-7	5497.33 ± 5.81	5994.67 ± 3.53	9.05	21,598.67 ± 3.53	21,800.67 ± 5.21	0.94	79.33 ± 2.91	100.67 ± 5.21	26.90
8	HA-8	5750.67 ± 4.67	5977.33 ± 4.06	3.94	20,798 ± 4.16	21,602 ± 4.16	3.87	60.67 ± 4.06	100.67 ± 5.21	65.93
9	HA-10	5971.33 ± 5.21	7157.33 ± 4.67	19.86	23,200.67 ± 4.06	23,801.33 ± 3.53	2.59	99.33 ± 4.06	99.33 ± 2.91	0.00
10	HA-11	5496.67 ± 5.21	5587.33 ± 4.06	1.65	17,382 ± 4.16	17,561.33 ± 4.67	1.03	38.67 ± 4.67	63.33 ± 4.67	63.77
11	K-12	5192.67 ± 5.21	5537.33 ± 5.21	6.64	16,039.33 ± 2.91	16,200.67 ± 5.21	1.01	140.67 ± 2.91	160.67 ± 5.21	14.22
12	K-13	6407.33 ± 3.53	6429.33 ± 5.21	0.34	23,799.33 ± 5.21	23,799.33 ± 5.21	0.00	202 ± 4.16	241.33 ± 3.53	19.47
13	K-14	4642.67 ± 4.67	4792.67 ± 3.53	3.23	23,001.33 ± 3.53	23,001.33 ± 2.4	0.00	121.33 ± 4.67	161.33 ± 6.96	32.97
14	K-15	5461.33 ± 5.21	5943.33 ± 4.67	8.83	21,600.67 ± 5.21	21,801.33 ± 4.67	0.93	78.67 ± 4.67	80.67 ± 2.91	2.54
15	K-16	4551.33 ± 4.67	4669.33 ± 2.91	2.59	23,199.33 ± 5.21	23,199.33 ± 4.06	0.00	139.33 ± 4.06	161.33 ± 5.81	15.79
16	K-17	4856.67 ± 4.67	5168.67 ± 4.06	6.42	22,198.67 ± 4.67	22,599.33 ± 4.06	1.80	218.67 ± 4.67	241.33 ± 3.53	10.36
17	K-19	5499.33 ± 4.67	5665.33 ± 4.67	3.02	9618 ± 4.16	15,980.67 ± 4.06	66.15	78.67 ± 4.67	100.67 ± 5.21	27.96
18	K-20	4771.33 ± 4.67	4934.67 ± 3.53	3.42	21,001.33 ± 3.53	21,398.67 ± 3.53	1.89	161.33 ± 4.67	177.33 ± 2.67	9.92
19	K-21	5372.67 ± 3.53	5387.33 ± 4.06	0.27	21,800.67 ± 4.06	22,000.67 ± 2.91	0.92	41.33 ± 5.81	77.33 ± 3.53	87.10
20	K-22	5306.67 ± 3.53	5397.33 ± 5.81	1.71	20,401.33 ± 4.67	20,599.33 ± 4.06	0.97	99.33 ± 4.06	118.67 ± 3.53	19.47
21	K-23	7337.33 ± 5.81	7753.33 ± 4.67	5.67	20,801.33 ± 3.53	21,401.33 ± 3.53	2.88	159.33 ± 5.21	199.33 ± 4.06	25.11
22	K-24	5424.67 ± 4.06	5547.33 ± 5.21	2.26	18,881.33 ± 4.67	18,881.33 ± 4.67	0.00	101.33 ± 4.67	159.33 ± 5.21	57.24
23	K-25	5143.33 ± 4.67	5422 ± 4.16	5.42	19,061.33 ± 4.67	19,419.33 ± 5.21	1.88	121.33 ± 5.81	165.33 ± 9.33	36.26
24	K-27	4710.67 ± 4.67	4791.33 ± 4.67	1.71	19,800.67 ± 2.91	20,199.33 ± 4.06	2.01	200.67 ± 5.21	241.33 ± 6.96	20.26
25	K-28	5734.67 ± 3.53	5914.67 ± 5.21	3.14	17,761.33 ± 3.53	18,161.33 ± 3.53	2.25	121.33 ± 6.96	147.33 ± 13.97	21.43
26	K-29	7280 ± 4.16	7715.33 ± 2.91	5.98	20,198.67 ± 3.53	20,201.33 ± 3.53	0.01	198.67 ± 3.53	321.33 ± 5.81	61.74
27	K-30	6170.67 ± 4.06	6648.67 ± 4.06	7.75	16,841.33 ± 4.67	17,479.33 ± 4.06	3.79	99.33 ± 4.06	139.33 ± 2.91	40.27
28	K-31	4893.33 ± 4.67	4989.33 ± 4.06	1.96	18,499.33 ± 4.06	18,641.33 ± 2.4	0.77	80.67 ± 5.21	121.33 ± 4.67	50.40
29	K-32	4823.33 ± 5.21	4990.67 ± 5.21	3.47	20,798 ± 4.16	21,201.33 ± 3.53	1.94	141.33 ± 3.53	181.33 ± 4.67	28.30
30	K-33	5512.67 ± 5.21	5944.67 ± 4.67	7.84	23,400.67 ± 4.06	23,600.67 ± 2.91	0.85	101.33 ± 4.67	140.67 ± 5.21	38.82
31	K-34	4844.67 ± 3.53	5117.33 ± 4.06	5.63	21,401.33 ± 3.53	22,199.33 ± 4.06	3.73	178 ± 4.16	200.67 ± 2.91	12.74
32	K-35	7625.33 ± 5.21	7773.33 ± 4.67	1.94	19,040.67 ± 4.06	19,340.67 ± 2.91	1.58	61.33 ± 4.67	82 ± 4.16	33.70
33	K-36	4843.33 ± 3.53	4980 ± 4.16	2.82	20,999.33 ± 5.21	21,398.67 ± 3.53	1.90	161.33 ± 5.81	182 ± 4.16	12.81

Table 2 (continued)

S. No	Genotype	Magnesium (ppm)			Calcium (ppm)			Sodium (ppm)		
		(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %	(Mean ± S.E)		Uptake %
		Control	Treated		Control	Treated		Control	Treated	
34	K-37	5907.33 ± 4.67	7109.33 ± 5.21	20.35	18,278.67 ± 3.53	18,679.33 ± 5.21	2.19	79.33 ± 4.06	101.33 ± 6.96	27.73
35	K-38	4866.67 ± 4.67	5127.33 ± 4.67	5.36	22,401.33 ± 3.53	22,798.67 ± 4.67	1.77	121.33 ± 5.81	151.33 ± 11.79	24.73
36	K-39	4930.67 ± 4.06	5167.33 ± 5.81	4.80	22,199.33 ± 2.91	22,599.33 ± 4.06	1.80	101.33 ± 3.53	138.67 ± 3.53	36.85
37	K-40	4984.67 ± 5.21	7405.33 ± 2.91	48.56	22,200.67 ± 2.91	22,399.33 ± 5.21	0.89	241.33 ± 3.53	260.67 ± 4.06	8.01
38	K-41	4883.33 ± 2.91	5285.33 ± 5.21	8.23	20,801.33 ± 2.4	21,801.33 ± 2.4	4.81	158.67 ± 3.53	160.67 ± 4.06	1.26
39	K-42	7415.33 ± 4.06	7507.33 ± 2.91	1.24	17,781.33 ± 2.4	17,939.33 ± 4.06	0.89	61.33 ± 4.67	99.33 ± 5.21	61.96
40	K-43	5726.67 ± 4.06	6017.33 ± 4.06	5.08	17,340.67 ± 2.91	17,981.33 ± 4.67	3.69	80.67 ± 4.06	100.67 ± 4.06	24.79
41	K-44	5592.67 ± 5.21	7658.67 ± 5.21	36.94	20,801.33 ± 2.4	21,201.33 ± 3.53	1.92	161.33 ± 5.81	281.33 ± 3.53	74.38
42	K-45	4857.33 ± 2.91	4971.33 ± 5.81	2.35	23,002 ± 4.16	23,398 ± 4.16	1.72	199.33 ± 4.06	219.33 ± 4.06	10.03
43	K-47	4835.33 ± 4.67	4965.33 ± 4.67	2.69	22,399.33 ± 4.06	22,799.33 ± 5.21	1.79	179.33 ± 4.06	200.67 ± 5.21	11.90
44	K-48	6484.67 ± 4.67	6750.67 ± 5.21	4.10	21,200.67 ± 2.91	21,801.33 ± 4.67	2.83	141.33 ± 4.67	141.33 ± 5.81	0.00
45	K-50	4838.67 ± 5.21	5045.33 ± 4.67	4.27	20,601.33 ± 3.53	20,798.67 ± 4.67	0.96	140.67 ± 2.91	161.33 ± 4.67	14.69
46	K-51	4823.33 ± 4.06	5047.33 ± 4.67	4.64	23,401.33 ± 3.53	23,802 ± 4.16	1.71	139.33 ± 2.91	161.33 ± 4.67	15.79
47	K-52	4785.33 ± 4.67	4953.33 ± 4.67	3.51	22,801.33 ± 3.53	23,001.33 ± 3.53	0.88	142 ± 4.16	221.33 ± 3.53	55.87
48	B-53	5066.67 ± 3.53	5169.33 ± 4.67	2.03	21,201.33 ± 4.67	21,801.33 ± 4.67	2.83	101.33 ± 5.81	141.33 ± 3.53	39.47
49	B-54	7444.67 ± 4.67	7595.33 ± 3.53	2.02	21,399.33 ± 5.21	22,000.67 ± 4.06	2.81	161.33 ± 4.67	179.33 ± 4.06	11.16
50	B-55	6649.33 ± 4.67	6753.33 ± 5.81	1.56	23,200.67 ± 2.91	23,801.33 ± 3.53	2.59	198.67 ± 4.67	279.33 ± 2.91	40.60
	Mean	5549.88	5880.41		20,499.59	20,992.27		132.72	167.32	
	C.D	2.570	2.727		2.873	2.707		3.074	3.346	
	SE(d)	1.293	1.372		1.445	1.362		1.547	1.684	
	SE(m)	0.914	0.970		1.022	0.963		1.094	1.191	
	CV	0.029	0.029		0.009	0.008		1.427	1.233	

Sodium (Na)

The highest level of sodium was observed in (control) genotype K-29 (198.67 ppm) and the lowest level in (control) genotype HA-11 (38.67 ppm) among all 50 rice genotypes (Table 2, Fig. 18S). The highest level of Na uptake was observed in K-21 (treated). The Na content was increased by 87.10% i.e. from 41.33 to 77.33 ppm. Following treatment, the Na concentrations in the genotypes HA-10 (99.33 ppm) and K-48 (141.33 ppm) did not change appreciably (Table 2, Fig. 6S). The p-value of Na was found to be0.0001, which is lesser than the alpha = 0.05 significance level. Therefore, data revealed that the mineral Na uptake of rice genotypes was statistically significant. The box plot and the corresponding p-value is shown in Fig. 2f.

Zinc (Zn)

Genotype (control) K-34 had the highest Zn concentration (10.17 ppm), while genotype (control) K-43 had the lowest concentration (1.27 ppm) (Table 1, Fig. 19S). In terms of Zn uptake, K-15 (treated) had the highest level of Zn uptake. The content of Zn was increased by 97.09%, from 1.03 to 2.03 ppm. The Zn concentration in the genotype K-37 (2.53 ppm) did not alter significantly following treatment (Table 1, Fig. 7S). Zn has a *p*-value of 0.0001, which is lower than the significance level of alpha = 0.05. Regarding Zn uptake, we may conclude that the treatment effect was significant among all 50 genotypes. The box plot and the corresponding p-value is shown in Fig. 2g.

Protein (%)

The highest level of protein content was found in (control) genotype B-54 (7.75%), while the lowest level was found in (control) genotype K-27 (4.06%) (Table 3, Fig. 20S). Genotype K-40 (treated) displayed maximum protein content among all 50 genotypes with 21.11% increase, i.e. 4.06 to 4.91%. While K-41 showed the lowest increase in protein content with only a 0.62% increase (Table 3, Fig. 8S). Conclusively, the impact of treatment on protein content was statistically significant, as the p-value of the test for protein was 0.0001, which was less than the significance level alpha = 0.05. The box plot and the corresponding p-value for protein are shown in Fig. 2h.

Starch (%)

The maximum level of starch content was found in (control) genotype K-37 (82.02%), and the minimum level was found in (control) genotype K-22 (75.55%) among all 50 rice genotypes (Table 3, Fig. 21S). The treated genotype K-24 displayed a maximum increase in starch content ranging from 80.00 to 80.88%, which is 1.108% increase. While B-55 showed the lowest increase in the content of starch among all 50 selected genotypes, with an increase of 0.141% (Table 3, Fig. 9S). The test for starch has a *p*-value of 0.0001, which was lower than the significance level of alpha = 0.05. In terms of starch content, we may conclude that the effect of treatment was statistically significant across all 50 selected rice genotypes. The box plot and the corresponding p-value for starch are shown in Fig. 2i.

Amylose (%)

Amylose content among control samples observed the highest level of amylose in genotype K-39 (25.45%) and the lowest level in genotype K-28 (18.77%) (Table 3, Fig. 22S). According to the findings, the treated genotype K-28 had the highest increase in amylose content (6.125%) from 18.77 to 19.93%. Genotype HA-11, on the other hand, had the lowest increase in amylose content among the 50 genotypes tested, with only a 1.43% rise (Table 3, Fig. 10S). Finally, the *p*-value for the amylose test was 0.0001, which was lower than the alpha = 0.05 significance limit. We may conclude that the effect of treatment on amylose content was statistically significant across all 50 rice genotypes. The box plot and the corresponding *p*-value for amylose are shown in Fig. 2j.

Amylopectin (%)

Genotype (control) K-28 (63.110%) had the highest content of amylopectin, whereas (control) genotype K-39 (52.490%) had the lowest content (Table 3, Fig. 23S). After the treatment, the amylopectin content showed a minimum decrease from 60.81 to 60.8% with (0.005%) in rice genotype HA-11. While in (treated) genotype K-45, there was a maximum decrease (1.142%) in amylopectin content, i.e. 58.94 to 58.27% (Table 3, Fig. 11S). The *p*-value of the test for amylopectin was 0.0001, which is lower than the significance level of alpha = 0.05. The findings infer a significant impact on the amylose content post-treatment across all 50 rice genotypes. The box plot and the corresponding p-value for amylopectin are shown in Fig. 2k.

Crude fibre (%)

Among all (control) 50 rice samples, K-36 (3.30%) demonstrated the highest crude fibre content, while a lower level was found in genotype K-45 (1.23%) (Table 3, Fig. 24S). The result showed a gradual increase in the crude fibre content of rice by nutrient application. Treated sample K-42 showed a maximum increase in crude fibre content that elevated from 1.23 to 1.69%, a 39.75% increase. While as, K-20 showed the lowest increase in crude fibre content

Table 3 Nutritional profile of different genotypes and increase in protein, starch and amylose content in response to fertilizer application

S. No	Genotype	Protein (%)			Starch (%)			Amylose (%)			Amylopectin (%)			Crude fibre (%)		
		(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase
		Control	Treated		Control	Treated		Control	Treated		Control	Treated		Control	Treated	
1	HA-1	5.04 ± 0.02	5.8 ± 0.02	14.937	80.42 ± 0.04	80.97 ± 0.03	0.68	22.34 ± 0.02	22.88 ± 0.02	2.447	58.09 ± 0.063	58.08 ± 0.009	-0.006	2.94 ± 0.03	3 ± 0.02	2.04
2	HA-2	7.24 ± 0.02	7.88 ± 0.02	8.794	79.65 ± 0.03	80.03 ± 0.02	0.485	22.45 ± 0.02	22.99 ± 0.02	2.406	57.2 ± 0.017	57.05 ± 0.003	-0.268	2.57 ± 0.02	2.96 ± 0.02	15.45
3	HA-3	4.05 ± 0.02	4.73 ± 0.03	16.969	77.76 ± 0.03	78.05 ± 0.02	0.382	21.22 ± 0.02	21.86 ± 0.03	2.984	56.53 ± 0.007	56.2 ± 0.007	-0.596	1.77 ± 0.03	2.05 ± 0.03	16.04
4	HA-4	6.05 ± 0.03	6.9 ± 0.02	14.105	76.29 ± 0.02	77 ± 0.03	0.922	19.04 ± 0.02	19.78 ± 0.03	3.886	57.25 ± 0.006	57.21 ± 0.003	-0.064	2.55 ± 0.03	2.89 ± 0.03	13.05
5	HA-5	6.24 ± 0.03	6.76 ± 0.02	8.445	81.23 ± 0.03	81.99 ± 0.03	0.940	23.01 ± 0.02	23.96 ± 0.02	4.129	58.22 ± 0.009	58.04 ± 0.007	-0.321	3.02 ± 0.02	3.31 ± 0.02	9.71
6	HA-6	4.23 ± 0.02	4.75 ± 0.02	12.283	80.59 ± 0.02	81.01 ± 0.02	0.525	22.46 ± 0.02	22.93 ± 0.02	2.123	58.13 ± 0.006	58.08 ± 0.007	-0.092	1.98 ± 0.02	2.02 ± 0.02	1.68
7	HA-7	7.34 ± 0.02	7.65 ± 0.03	4.316	77.78 ± 0.02	78.45 ± 0.03	0.861	25.04 ± 0.02	25.83 ± 0.02	3.155	52.74 ± 0.006	52.62 ± 0.01	-0.228	2.83 ± 0.02	2.99 ± 0.03	5.90
8	HA-8	4.79 ± 0.03	5 ± 0.02	4.387	81.66 ± 0.03	82.11 ± 0.03	0.551	20.22 ± 0.03	20.87 ± 0.02	3.248	61.45 ± 0.003	61.24 ± 0.006	-0.336	1.86 ± 0.02	2 ± 0.02	7.16
9	HA-10	5.55 ± 0.03	5.79 ± 0.03	4.202	80.18 ± 0.02	80.81 ± 0.03	0.777	23.22 ± 0.02	23.85 ± 0.02	2.742	56.97 ± 0.003	56.95 ± 0.007	-0.023	2.32 ± 0.03	2.79 ± 0.02	19.94
10	HA-11	7.54 ± 0.02	8 ± 0.02	6.103	80.51 ± 0.02	80.79 ± 0.03	0.348	19.7 ± 0.02	19.99 ± 0.02	1.438	60.81 ± 0.006	60.81 ± 0.018	-0.005	2.85 ± 0.03	2.97 ± 0.02	4.21
11	K-12	6.1 ± 0.03	6.67 ± 0.02	9.230	78.92 ± 0.02	79.22 ± 0.03	0.372	23.08 ± 0.02	23.87 ± 0.02	3.452	55.85 ± 0.009	55.34 ± 0.009	-0.901	1.95 ± 0.02	2.22 ± 0.02	13.68
12	K-13	5.05 ± 0.03	5.79 ± 0.03	14.663	80.61 ± 0.03	80.98 ± 0.02	0.467	23.44 ± 0.02	23.83 ± 0.02	1.635	57.16 ± 0.013	57.16 ± 0.003	-0.012	2.87 ± 0.02	3 ± 0.02	4.65
13	K-14	6.66 ± 0.03	6.99 ± 0.02	4.957	79.81 ± 0.02	80.45 ± 0.03	0.806	20.17 ± 0.02	20.82 ± 0.02	3.223	59.64 ± 0.006	59.63 ± 0.012	-0.011	1.96 ± 0.02	2.02 ± 0.02	3.41
14	K-15	7.05 ± 0.02	7.44 ± 0.02	5.629	81.31 ± 0.02	81.97 ± 0.02	0.812	25.12 ± 0.02	25.78 ± 0.02	2.654	56.2 ± 0.007	56.19 ± 0.006	-0.012	1.91 ± 0.03	2.22 ± 0.03	16.20
15	K-16	7.57 ± 0.02	7.93 ± 0.02	4.754	78.92 ± 0.02	79.53 ± 0.03	0.773	23.02 ± 0.02	23.84 ± 0.02	3.562	55.89 ± 0.009	55.68 ± 0.009	-0.376	2.44 ± 0.02	2.67 ± 0.03	9.44
16	K-17	4.07 ± 0.03	4.33 ± 0.02	6.557	80.4 ± 0.03	81.05 ± 0.02	0.817	22.12 ± 0.02	22.78 ± 0.02	2.984	58.28 ± 0.01	58.28 ± 0.003	-0.006	2.45 ± 0.02	2.88 ± 0.02	17.26
17	K-19	5.08 ± 0.03	5.23 ± 0.02	2.820	82.02 ± 0.03	82.44 ± 0.02	0.512	22.14 ± 0.02	22.79 ± 0.02	2.905	59.88 ± 0.013	59.65 ± 0.007	-0.373	1.91 ± 0.03	2.03 ± 0.03	6.29
18	K-20	6.01 ± 0.03	6.32 ± 0.02	5.214	76.38 ± 0.03	76.92 ± 0.02	0.707	21.15 ± 0.02	21.7 ± 0.02	2.569	55.22 ± 0.012	55.22 ± 0.006	-0.006	3 ± 0.03	3.02 ± 0.03	0.89
19	K-21	7.04 ± 0.02	7.46 ± 0.03	5.868	78.34 ± 0.03	78.84 ± 0.03	0.638	25.03 ± 0.02	25.98 ± 0.02	3.823	53.32 ± 0.007	52.86 ± 0.015	-0.857	3.2 ± 0.03	3.33 ± 0.03	4.28
20	K-22	6.72 ± 0.02	7 ± 0.02	4.169	75.55 ± 0.03	75.76 ± 0.03	0.282	20.22 ± 0.02	20.83 ± 0.02	3.050	55.33 ± 0.012	54.93 ± 0.01	-0.729	1.88 ± 0.03	2.04 ± 0.02	8.14
21	K-23	4.45 ± 0.02	4.87 ± 0.03	9.281	80.27 ± 0.02	81.1 ± 0.02	1.042	19.02 ± 0.02	19.87 ± 0.02	4.451	61.25 ± 0.009	61.24 ± 0.007	-0.016	2.45 ± 0.02	2.87 ± 0.02	16.85
22	K-24	4.33 ± 0.02	4.46 ± 0.03	2.925	80 ± 0.03	80.88 ± 0.02	1.108	22.02 ± 0.02	22.91 ± 0.02	4.042	57.98 ± 0.012	57.98 ± 0.003	-0.006	2.79 ± 0.02	2.96 ± 0.02	6.10
23	K-25	6.63 ± 0.03	6.88 ± 0.02	3.773	80.22 ± 0.02	80.87 ± 0.02	0.802	23.03 ± 0.02	23.81 ± 0.02	3.386	57.19 ± 0	57.05 ± 0.003	-0.239	2.82 ± 0.03	2.94 ± 0.03	4.14
24	K-27	4.06 ± 0.02	4.27 ± 0.03	5.168	80.4 ± 0.02	81.01 ± 0.03	0.750	20.23 ± 0.02	20.84 ± 0.02	3.016	60.18 ± 0.003	60.17 ± 0.012	-0.011	1.61 ± 0.02	1.89 ± 0.03	17.36
25	K-28	5.95 ± 0.02	6 ± 0.02	0.896	81.89 ± 0.02	82.43 ± 0.03	0.668	18.78 ± 0.02	19.93 ± 0.02	6.125	63.11 ± 0	62.51 ± 0.012	-0.956	1.99 ± 0.03	2.23 ± 0.02	11.71
26	K-29	4.92 ± 0.03	5.01 ± 0.02	1.828	78.81 ± 0.03	79.21 ± 0.02	0.516	21.1 ± 0.02	21.94 ± 0.02	3.949	57.7 ± 0.003	57.28 ± 0.009	-0.739	2.88 ± 0.03	2.98 ± 0.02	3.24
27	K-30	6.06 ± 0.03	6.76 ± 0.02	11.490	77.72 ± 0.03	78.04 ± 0.03	0.412	22.05 ± 0.02	22.55 ± 0.02	2.237	55.66 ± 0.015	55.49 ± 0.006	-0.311	2.55 ± 0.02	2.9 ± 0.02	13.87
28	K-31	4.28 ± 0.02	4.31 ± 0.02	0.701	79.69 ± 0.03	80.45 ± 0.02	0.945	22.13 ± 0.02	22.89 ± 0.03	3.435	57.57 ± 0.013	57.56 ± 0.006	-0.012	1.53 ± 0.03	1.95 ± 0.02	27.95
29	K-32	5.59 ± 0.02	5.92 ± 0.02	5.781	79.13 ± 0.02	79.87 ± 0.04	0.935	23.02 ± 0.02	23.76 ± 0.02	3.244	56.12 ± 0.009	56.11 ± 0.017	-0.012	3.11 ± 0.02	3.36 ± 0.02	8.05
30	K-33	4.11 ± 0.03	4.23 ± 0.02	3.084	80.25 ± 0.03	80.85 ± 0.02	0.748	20.26 ± 0.02	20.86 ± 0.02	2.978	59.99 ± 0.009	59.98 ± 0.009	-0.006	1.44 ± 0.03	1.94 ± 0.03	34.18
31	K-34	6.66 ± 0.02	6.82 ± 0.03	2.351	80.31 ± 0.02	80.92 ± 0.02	0.760	22.07 ± 0.02	22.69 ± 0.02	2.810	58.24 ± 0.006	58.23 ± 0.006	-0.017	3.21 ± 0.02	3.45 ± 0.03	7.47
32	K-35	7.38 ± 0.02	7.94 ± 0.02	7.543	78.75 ± 0.02	79.03 ± 0.03	0.351	19.32 ± 0.03	19.99 ± 0.02	3.469	59.44 ± 0.009	59.04 ± 0.009	-0.662	2.28 ± 0.03	2.68 ± 0.03	17.86
33	K-36	6.81 ± 0.03	6.96 ± 0.03	2.202	81.06 ± 0.03	81.84 ± 0.02	0.954	23.09 ± 0.02	23.87 ± 0.02	3.379	57.98 ± 0.009	57.97 ± 0.006	-0.011	3.3 ± 0.02	3.85 ± 0.02	16.45
34	K-37	5.87 ± 0.02	5.96 ± 0.02	1.476	82.41 ± 0.02	82.88 ± 0.02	0.570	22.22 ± 0.02	22.7 ± 0.02	2.161	60.19 ± 0	60.18 ± 0.006	-0.017	2.46 ± 0.02	2.93 ± 0.03	19.11
35	K-38	4.07 ± 0.02	4.45 ± 0.03	9.419	79.18 ± 0.03	79.76 ± 0.02	0.737	20.13 ± 0.02	20.83 ± 0.02	3.477	59.04 ± 0.009	58.93 ± 0.003	-0.198	2.55 ± 0.02	2.97 ± 0.02	16.49
36	K-39	7.11 ± 0.03	7.64 ± 0.02	7.501	77.95 ± 0.03	78.31 ± 0.03	0.470	25.46 ± 0.02	25.87 ± 0.02	1.611	52.49 ± 0.006	52.45 ± 0.015	-0.083	2.71 ± 0.03	2.96 ± 0.02	9.21
37	K-40	4.06 ± 0.02	4.91 ± 0.03	21.118	75.45 ± 0.02	76 ± 0.03	0.733	21.07 ± 0.02	21.85 ± 0.02	3.734	54.38 ± 0.006	54.15 ± 0.009	-0.429	1.86 ± 0.03	2.04 ± 0.03	10.05

Table 3 (continued)

S. No	Genotype	Protein (%)			Starch (%)			Amylose (%)			Amylopectin (%)			Crude fibre (%)		
		(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase	(Mean ± S.E)		% increase
		Control	Treated		Control	Treated		Control	Treated		Control	Treated		Control	Treated	
38	K-41	6.98 ± 0.03	7.03 ± 0.02	0.621	77.32 ± 0.03	78 ± 0.03	0.879	19.03 ± 0.02	19.88 ± 0.02	4.431	58.28 ± 0.012	58.12 ± 0.01	-0.280	1.88 ± 0.02	2.55 ± 0.03	35.88
39	K-42	4.26 ± 0.02	4.38 ± 0.02	2.815	80.52 ± 0.03	80.86 ± 0.03	0.426	21.32 ± 0.02	21.67 ± 0.03	1.642	59.2 ± 0.015	59.2 ± 0.003	-0.011	1.32 ± 0.02	1.84 ± 0.02	39.75
40	K-43	5.78 ± 0.02	6.02 ± 0.02	4.095	78.7 ± 0.02	79.46 ± 0.02	0.961	22.22 ± 0.02	22.98 ± 0.02	3.420	56.48 ± 0.003	56.47 ± 0.003	-0.006	3.33 ± 0.02	3.45 ± 0.03	3.61
41	K-44	6.33 ± 0.03	6.77 ± 0.03	7.004	75.53 ± 0.02	76.01 ± 0.03	0.635	19.29 ± 0.02	19.78 ± 0.02	2.541	56.25 ± 0.003	56.24 ± 0.009	-0.018	2.94 ± 0.02	3.04 ± 0.03	3.41
42	K-45	4.1 ± 0.02	4.37 ± 0.02	6.580	81 ± 0.03	81.14 ± 0.03	0.173	22.06 ± 0.02	22.87 ± 0.03	3.687	58.94 ± 0.012	58.27 ± 0.006	-1.142	1.23 ± 0.02	1.69 ± 0.02	37.13
43	K-47	5.14 ± 0.03	5.77 ± 0.02	12.322	78.67 ± 0.02	79.34 ± 0.03	0.860	19.01 ± 0.02	19.74 ± 0.02	3.804	59.65 ± 0.009	59.61 ± 0.015	-0.078	3.23 ± 0.02	3.35 ± 0.02	3.93
44	K-48	7.16 ± 0.03	7.72 ± 0.03	7.868	81.44 ± 0.02	81.89 ± 0.03	0.553	23.13 ± 0.03	23.92 ± 0.02	3.416	58.31 ± 0.01	57.97 ± 0.012	-0.583	2.54 ± 0.02	2.87 ± 0.02	13.14
45	K-50	6.93 ± 0.02	7.05 ± 0.03	1.781	76.46 ± 0.02	77 ± 0.02	0.715	20.11 ± 0.03	20.84 ± 0.02	3.596	56.34 ± 0.003	56.17 ± 0.007	-0.314	2.64 ± 0.03	2.91 ± 0.02	10.49
46	K-51	7.12 ± 0.03	7.73 ± 0.02	8.571	79.65 ± 0.02	80.22 ± 0.03	0.720	19.18 ± 0.02	19.76 ± 0.03	3.025	60.47 ± 0.006	60.46 ± 0.003	-0.011	3.29 ± 0.02	3.43 ± 0.03	4.25
47	K-52	4.98 ± 0.02	5.04 ± 0.02	1.273	76.47 ± 0.02	76.87 ± 0.03	0.523	22.05 ± 0.02	22.46 ± 0.02	1.829	54.42 ± 0.006	54.42 ± 0.003	-0.006	2.76 ± 0.02	2.89 ± 0.02	4.83
48	B-53	6.06 ± 0.02	6.96 ± 0.02	14.788	80.28 ± 0.02	80.98 ± 0.02	0.872	23.16 ± 0.02	23.87 ± 0.02	3.066	57.13 ± 0.007	57.12 ± 0.009	-0.018	1.82 ± 0.03	2.03 ± 0.03	11.52
49	B-54	7.75 ± 0.03	7.99 ± 0.02	3.095	79.44 ± 0.02	80.02 ± 0.03	0.730	25.02 ± 0.02	25.78 ± 0.02	3.038	54.43 ± 0.003	54.25 ± 0.009	-0.331	2.66 ± 0.02	2.95 ± 0.02	10.92
50	B-55	5.92 ± 0.02	6.07 ± 0.03	2.532	75.9 ± 0.03	76 ± 0.02	0.141	22.02 ± 0.03	22.67 ± 0.02	2.936	53.87 ± 0.003	53.33 ± 0.003	-1.002	2.28 ± 0.03	2.86 ± 0.02	25.62
	Mean	5.8	6.17		79.26	79.79		21.73	22.41		57.52	57.38		2.43	2.7	
	CD	0.018	0.017		0.017	0.017		0.013	0.013		0.013	0.013		0.016	0.016	
	SE(d)	0.009	0.008		0.008	0.008		0.006	0.006		0.006	0.006		0.008	0.008	
	SE(m)	0.006	0.006		0.006	0.006		0.005	0.005		0.005	0.005		0.006	0.006	
	CV	0.186	0.168		0.013	0.013		0.036	0.035		0.010	0.010		0.393	0.376	

with only a 0.89% increase (Table 3, Fig. 12S). We found that there was a significant impact on the crude fibre content post-treatment, which was statistically significant (*p*-value = 0.0001). The box plot and the corresponding *p*-value for crude fibre, is shown in Fig. 2l.

Correlation analysis of minerals and nutrients

The correlation study for uptake of minerals and nutrients viz. phosphorous, iron, potassium, magnesium, calcium, sodium, zinc, protein, starch, amylose, amylopectin and crude fibre shows interesting results (Figs. 3 and 4).

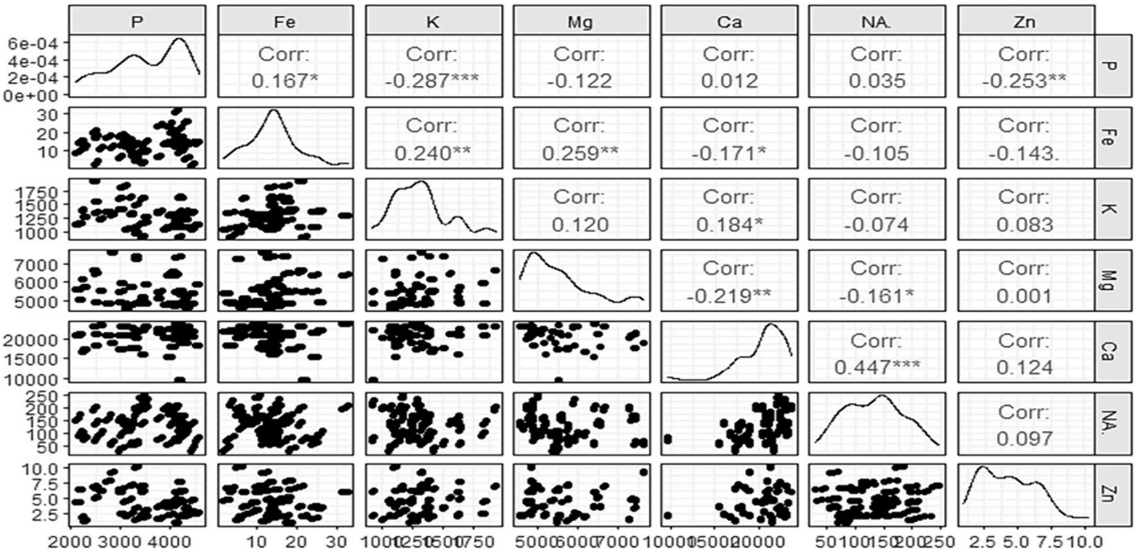

Fig. 3 Correlation chart among 7 minerals for all selected 50 (control) rice genotypes. The *stars* (*) indicate the significance of the correlation coefficient. The absence of stars indicates that the variable is not statistically significant, while one, two or three *stars* mean that the corresponding variable is significant at 10%, 5% and 1% levels. respectively

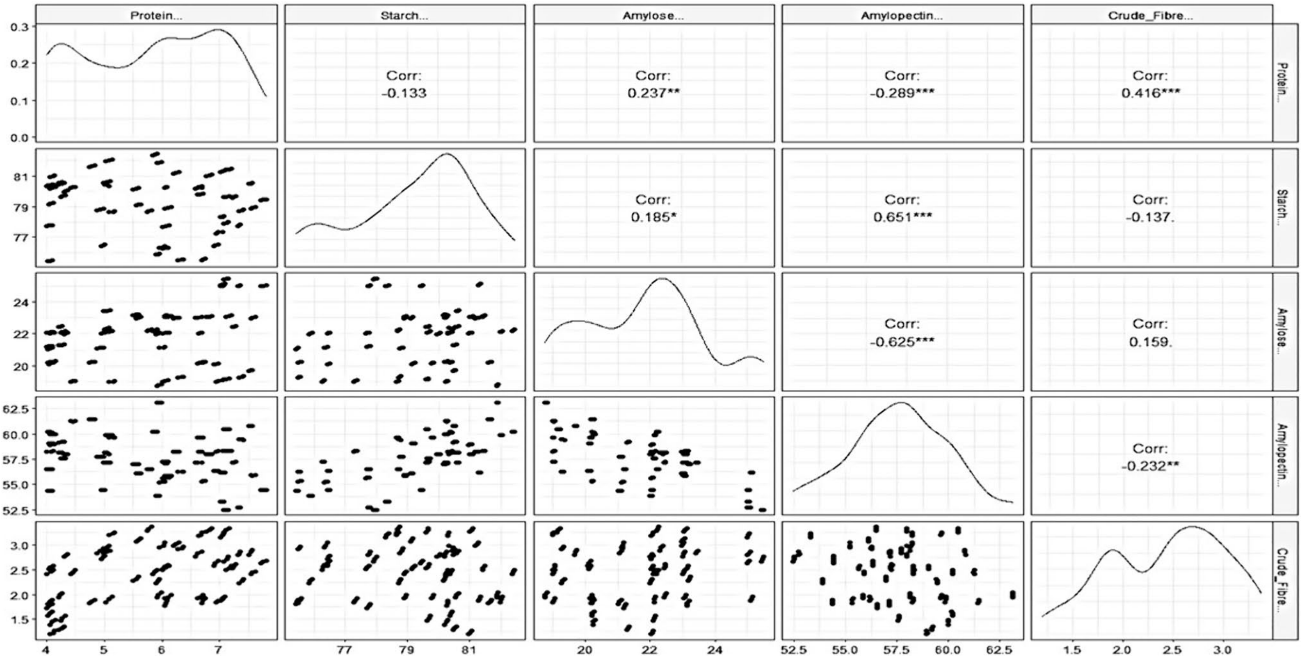

Fig. 4 Correlation chart among 5 minerals for all selected 50 (control) rice genotypes. The *stars* (*) indicate the significance of the correlation coefficient. The absence of stars indicates that the variable is not statistically significant, while one, two or three *stars* mean that the corresponding variable is significant at 10%, 5% and 1% levels. respectively

There was a significant positive correlation between sodium and calcium (correlation coefficient of 0.447***), signifying that when sodium concentration increases, the concentration of calcium also increases, and vice versa at $p < 0.01$ (Fig. 3). Similarly, iron has a correlation coefficient of 0.259** and 0.240** with magnesium and potassium, respectively ($p < 0.05$). A significant negative correlation was found between phosphorous and potassium (−0.287***) at 1% level of significance. Similarly, phosphorous and zinc have a correlation coefficient of −0.253**, signifying that increase in phosphorus concentration decreases zinc and vice versa. Meanwhile, there is no significant association of phosphorous with magnesium (−0.122), calcium (0.012) and sodium (0.035); iron with sodium (−0.105) and zinc (−0.143); potassium with magnesium (0.120), sodium (−0.074) and zinc (0.083); magnesium with zinc (0.001); calcium with zinc (0.124) and sodium with zinc (0.097) (Fig. 3).

There is a significant positive correlation of amylopectin with starch (0.651***) and protein with crude fibre (0.416***). Similarly, a correlation coefficient of 0.237** between protein and amylose indicates that both protein and amylose content increase together (Fig. 4). A significant negative correlation was found for protein with amylopectin (−0.289***) and amylose with amylopectin (−0.625***). Similarly, the correlation coefficient of −0.232** between crude fibre and amylopectin indicates that when crude fibre content increases, the content of amylopectin will decrease and vice versa ($p < 0.05$). However, no association was recorded for protein with starch (−0.133); amylose with crude fibre (0.159) and crude fibre with starch (0.137).

Comparison of mineral uptake and nutrient content with genetic diversity

The mineral composition and nutritional quality of the rice germplasm set was examined to find connection between the genetic diversity groups reported earlier (Pandita et al. 2023) and their nutritional profiles. With respect to nutrient profiling, our findings revealed that most of the fertilizer-responsive genotypes were concentrated in cluster I (HA-4, HA-5, HA-6, HA-7, HA-8, K-39, K-40, K-41, K-42, K-43, K-44, K-45, K-47, K-48, K-50, K-52, B-53, B-54, B-55). Overall, in cluster I, all the seven minerals have demonstrated more uptake of minerals compared to untreated genotypes, i.e. 7.20% more uptake in P, 23.71% for Fe, 9.40% for K, 7.92% for Mg, 2.26% for Ca, 29.15% for Na and 21.37% for Zn. In cluster II the uptake for minerals like P, Fe, K, Mg, Ca, Na and Zn was found more in comparison to untreated genotypes i.e. 4.66%, 32.93%, 11.02%, 4.36%, 5.90%, 26.84% and 13.57%, respectively. Further, in cluster III, 7.23% more uptake was found for P, 33.04% for Fe, 11.08% for K, 5.13% for Mg, 1.46% for Ca, 33.19% for Na and 27.05% for Zn in contrast to untreated samples.

An intra-cluster nutrient comparison was also performed to determine the best genotypes demonstrating the most cumulative nutrient content. Among cluster I, genotype HA-4 showed higher nutrient content for three nutrients (Protein, Starch and Amylose) followed by six more genotypes that displayed nutrient content accumulation for different nutrients viz. genotype K-47 and K-40 showed each for protein and amylose, genotype K-45 and K-41 displayed each for amylose and crude fibre, genotype HA-6 showed for protein and amylopectin and genotype K-42 demonstrated for amylopectin and crude fibre. Intra-cluster mineral comparison was also undertaken to find the genotypes that show maximum cumulative mineral uptake. Among cluster I, genotype K-44 has shown higher mineral uptake for four minerals (Fe, K, Mg and Na), genotype HA-8 has displayed higher mineral uptake for four minerals (Ca, Zn, Na, P) followed by genotype HA-4, which showed more mineral uptake for three minerals (P, K, Ca).

Discussion

Rice (*Oryza sativa*) is the staple food for over half of the world's population and the global rice consumption is estimated to be 54 kg/ capita/year. China and India account for more than half of the global rice consumption (Muthayya et al. 2014). India has the largest rice cultivation area (44 million ha) and continues to rank second in rice production. Rice accounts for 43% of total food and 55% of cereal production in the country. Almost 31% of the calories of the Indian diet are estimated to be supplied through rice. Rice plays a pivotal role in the Indian economy; thus, self-sufficiency in rice production and continued upliftment in economic conditions have resulted in changing lifestyles and increased awareness among consumers to improve and diversify diets, including the quality of rice consumed.

Western Himalayas are a storehouse of high-altitude cold-tolerant rice genotypes classified into group VI (Glaszmann 1987; Husaini et al. 2021). In India, after the Green revolution in the late 1960s, high-yielding rice varieties became popular, leading to the genetic erosion of many useful rice genotypes in the region. These rice genotypes are very important for breeding climate-smart varieties for the region (Ashraf et al. 2016), especially because these differ from sub-tropical and tropical rice genotypes in the rest of India (Ashraf et al. 2017). Considering the urgent need to make rice cultivation more remunerative, the present study has screened the germplasm resources of Western-Himalayan region to identify and promote 'high-value rice genotypes'. These genotypes with higher nutrient profiles can be supplied to common people consuming rice diets and can provide genes for crop improvement through smart breeding (Husaini et al. 2013). The present study focussed on the nutritional composition of fifty rice genotypes of the region and the response of these genotypes to mineral uptake for rice grain bio-fortification. Analytical results of minerals in the fifty rice genotypes are shown in Tables 1 & 2, and nutrients in Table 3.

Mineral content

Micronutrient malnutrition affects over three billion people, and 3.1 million children die each year as a result of it (Gearing 2015) and the numbers are gradually increasing. The situation in developing countries is more alarming than in developed countries. Vitamins and minerals are now being coated on rice kernels to supplement the requirement for a healthy diet. However, mineral biofortification may not be affordable for a larger section of the population in developing countries (Tripathy et al. 2017). Therefore, higher mineral containing rice varieties need to be identified and used in breeding programmes.

The human body requires zinc and iron for enzymatic processes and haemoglobin production. Symptoms of zinc deficiency include diarrhoea, weight loss and illness. Iron deficiency can harm one’s health, causing anaemia and symptoms such as brittle hair, brittle fingernails and fatigue. In the present study, the order of the mean concentration of micronutrient element was Fe ˃ Zn. Iron concentrations range from 2.27 to 31.93 ppm and zinc from 1.27 to 10.17 ppm. This shows that there is a huge variation between the genotypes with respect to Fe and Zn content. The concentration of iron in our study was higher, and concentration of zinc was lower than the concentration observed in a previous study (Srinuttrakul and Busamongkol 2014), where the concentration of iron varied from 5.10 to 9.75 mg kg^{-1} and zinc from 22.5 to 32.7 mg kg^{-1}. The concentration observed for Zn and Fe in an earlier study on different rice genotypes (Tyagi et al. 2020), was reported as 117 µg g^{-1} Zn and 2.5 µg g^{-1} Fe.

Rice provides essential minerals such as potassium (K), phosphorus (P), manganese (Mn), magnesium (Mg), calcium (Ca), and sodium (Na), each crucial for maintaining human health. Potassium supports normal metabolic, cellular, tissue, and organ functions, including muscle growth and cardiac activity. Phosphorus aids in regulating internal water balance and nutrient metabolism. Manganese is vital for optimal brain and nerve function, while copper facilitates enzyme production necessary for bodily processes. Magnesium participates in more than 300 enzyme reactions, contributing to muscle and nerve function, blood pressure regulation, and immune system support. Calcium contributes to bone and dental health, blood clotting, muscular contraction, and the regulation of heart rhythms and nerve functions. Sodium plays a role in nerve impulse transmission, muscle contraction, and maintaining proper fluid and mineral balance in the body. Understanding the diverse roles of these minerals in rice underscores their importance in human nutrition and physiological well-being. In the present study, the concentration of sodium was remarkably less compared with that of other macronutrients in rice genotypes. The order of the mean concentration of element was Ca ˃ Mg ˃ P ˃ K ˃ Na. The concentrations of calcium varied from 9618.00 to 23,799.33 ppm, magnesium from 4551.33 to 7625.33 ppm, phosphorous from 2133.33 to 4553.33 ppm, potassium from 921.33 to 1921.33 ppm and sodium from 38.67 to 198.67 ppm. The concentration of phosphorous, potassium, magnesium and calcium was higher than that observed in the previous study (Srinuttrakul and Busamongkol 2014), where the phosphorous concentration varied from 3024 to 3830 mg kg^{-1}, potassium from 1404 to 1927 mg kg^{-1}, magnesium from 980 to 1284 mg kg^{-1} and calcium from 72 to 128 mg kg^{-1}. In the present study, the concentration of Ca, K, Zn, Fe and Na was higher than the concentration observed in a previous study (Verma and Srivastav 2017) where the concentration of calcium was 98.75 mg/kg, potassium was 500.00 mg/kg, zinc was 17.00 mg/kg, iron was 31.50 mg/kg, and sodium was 68.85 mg/kg.

Nutrient content

Rice is considered as the queen of cereals due to its nutritional quality and higher digestibility (Anjum et al. 2007). The rice grain is made up of 12% water, 75–80% carbohydrates, and just 7% protein containing all of the essential amino acids. Its protein is highly digestible (93%) and has a high biological value (74%) and protein efficiency ratio (2.02–2.04%) due to the presence of a greater lysine content (about 4%) (Eggum 1969, 1973, 1977; Juliano 1993; Juliano et al. 1971).

Starch, amylose and amylopectin

Starch, which is made up of amylose (linear) and amylopectin (branched), is the major carbohydrate in rice. Amylose content is considered the most important character for predicting rice cooking and processing behaviour. The ratio of two types of starches, amylose and amylopectin, influences cooking characteristics, gelatinization behaviour, pasting properties and glycemic index of rice (Frei et al. 2003; Juliano 1985; Lamberts et al. 2009; Larsen et al. 2000). Amylose is almost absent from the waxy (glutinous) rice cultivars that do not expand in volume and remain firm when cooked, while high amylose rice grains show high volume expansion and a high degree of flakiness. Short-grain rice typically contains the lowest levels of amylose and the highest of amylopectin (Thomas et al. 2013). The present study found the minimum starch content in genotype K-22 (75.55%) and the maximum in genotype K-37 (82.02%). In some previous studies, the starch content in the rice ranged between 72.92 and 88.18% (Susiyanti et al. 2020) and 73.6 to 83.7% (Devi et al. 2015). The starch content reported of Shalimar rice-1 was reported earlier as 79.30% (Ashraf et al. 2017), and of Jhelum was 78.89%, which is similar (80.6 and 78.92%) to that reported in the present study.

In the present investigation, the lowest level of amylose content was found in genotype K-28 (18.77%) and highest in K-39 (25.45%). The amylose content reported in a previous study (Susiyanti et al. 2020) ranged from 17 to 24%. The results on amylose content in the present study are consistent with those of Ashraf et al. (Ashraf et al. 2017), who reported its range from 18.23 to 24.43%. In the present study, Pusa sugandh-3 had 22.02% amylose content, which is lower than that reported earlier 23.56% (Ashraf et al. 2017) and 26.78% (Husaini et al. 2009). Basmati-370, a thin long aromatic rice variety similar to Pusa sugandh-3, has an amylose content of 19.9% (Rosniyana et al. 1995). The amylose content of rice varieties has been reported to vary from 16.8 to 25.05% (Deepa et al. 2008; Subudhi et al. 2013). This variation in amylose content may be due to variations in the temperature during the grain ripening stage (Resurrection et al. 1979). In addition, the amylose content is also influenced by nitrogen fertilization, whereby the value decreases slightly with nitrogen fertilization (Paule et al. 1979).

In the present study, the lowest level of amylopectin content was found in genotype K-39 (52.49%) and the highest in K-28 (63.11%), which is in coherence with a previous study (Susiyanti et al. 2020), where amylopectin ranged from 54 to 70%. The ratio of amylose/amylopectin determines the texture of rice. The smaller the amylose content or the higher the amylopectin content, the stickier the rice will be. However, the rice genotypes studied in the present investigation possess low amylopectin, which indicates that these are not glutinous.

Protein

Protein content of more than 10% is considered ‘high’ (Resurrection et al. 1979). In the present study, the lowest protein content was observed in genotype K-27 (4.06%) and the highest in B-54 (7.75%), which is lower than many other varieties studies earlier (Devi et al. 2015; Guha and Mitra 1963). The protein content observed in our study was lower than the protein content (7.32–10.93%) reported for several Indonesian rice varieties (Susiyanti et al. 2020). Some varieties from Himachal Pradesh, India have 6.61 to 7.28% of total crude protein (Singh et al. 1998). Indigenous cultivars of the north-eastern hill states of India possess high protein content ranging from 6.14 to 12.07% (Devi et al. 2008; Thongbam et al. 2010). Similar crude protein content was reported (6–12.6%) in another study on 300 improved rice varieties in India (Govindaswami et al. 1969). The range of protein content in the present study is similar to that obtained earlier (Pathmanathapillai et al. 2016), which ranged from 5.96 to 7.00%. Protein content in several Pakistani rice genotypes has been reported to range from 7.38 to 8.13% (Awan 1996), which is higher than those observed for the northwestern Himalayas in the present study. The protein content in Jhelum and Shalimar rice 1 was reported as 6.55 and 8.57%, respectively (Ashraf et al. 2017) and, 8.42 and 8.65%, respectively (Lone et al. 2013) which is higher than the protein content observed in the same genotypes (6.1 and 5.05%), respectively, in the present study. This could be due to the variation in fertilizer application and soil nutrient composition. Very low crude protein content between 1.17–7.94 and 1.6–7.9% were reported in a set of rice varieties earlier (Oko et al. 2012; Oko and Ugwu 2011), which is significantly lower than many published studies including the present study.

In the present study, the protein content of basmati types SKUA-420, SKUA- 494 and Pusa Sugandh-3 ranged between 5.92 and 7.75%. In another study, the protein content of basmati types ranged from 7.75 to 8.96% in Pusa basmati-1 and Tarori basmati (Subudhi et al. 2013). A study on six different rice varieties marketed in Penang, Malaysia (both locally grown and imported) found that the protein content ranged between 5.96 and 8.16%, with Pakistani Basmati showing 7.75% (Thomas et al. 2013). Basmati-370 has a high protein content of 9.84% (Rosniyana et al. 1995) compared to all the other basmati varieties. These studies show that the nutritional value of rice varies with different varieties, soil fertility, fertilizer application and other environmental factors.

Dietary fibre

Rice also consists of crude fibre, a part of insoluble fibre found in the edible portion of the plant cell wall. The main health benefit of crude fibre is that it facilitates regular bowel movement. Fibre in the diet increases the bulk of faeces, resulting in a laxative action in the gut. The fibre content of well-milled rice is 0.5–1.0% (Oko and Onyekwere 2010). Fibre content is very low in rice as the maximum amount of fibre is present in the husk, which is removed during milling. However, in some rice types adequate amounts of dietary fibre are also found in rice grains (Aune et al. 2011), thus rendering rice as a healthy food (Thomas et al. 2015). Thomas et al. worked on six different rice varieties of Malaysia (white local, Brown, Bario, Black, Glutinous and Basmati rice types) and observed that the fibre content varied in the range of 7.07 to 8.47% (Thomas et al. 2013). Similarly, the crude fibre content in the rice samples in a previous study ranged between 8.91 and 24.51% (Susiyanti et al. 2020). Deepa et al. have reported that the total dietary fibre content in Njavara was 8.08%, which is 34 to 44% higher than Jyoti (5.82%) and IR-64 (4.96%) (Deepa et al. 2008). In these studies, the rice genotypes had a much higher crude fibre content than was observed in the present study. In the present study, the highest crude fibre content was found in K-36 (3.30%), and the lowest was found in K-45 (1.23%). Our results are in coherence with the results of the previous study (Ashraf et al. 2017), where the fibre content ranged from 1.38 to 3.31% and (Edeogu et al. 2007), where crude fibre content ranged from 1.93 to 4.3%. The crude fibre content in the present study is higher than that reported by (Devi et al. 2015) (0.22–0.95%). In the present study, the fibre content of the long-grain aromatic variety Pusa sugandh-3 was 2.28%, which is a little lower than the 2.94% reported in a previous study (Ashraf et al. 2017). These results show that there is a huge variation in the fibre content amongst the rice genotypes of different geographical regions.

Conclusion

The present study revealed that the rice germplasm of the Western Himalayan region of Kashmir is diverse in terms of nutritional profiles and fertilizer responsiveness. Overall, the study underscored the significance of fertilizer application treatments in modulating mineral uptake and nutrient content in rice genotypes, which can have implications for agricultural practices aimed at enhancing crop nutritional quality and productivity.

The fertilizer treatment had a significant effect on the uptake of minerals and nutrients among most of the tested rice genotypes, irrespective of the altitude where they are generally cultivated. Notably, there were positive correlations observed between sodium and calcium, iron and magnesium, iron and potassium, as well as protein and crude fiber. Conversely, there were negative correlations observed between phosphorous and potassium, phosphorous and zinc, protein and amylopectin, and crude fiber and amylopectin. The comparison of mineral uptake and nutrient content with genetic diversity groups suggested that fertilizer-responsive genotypes primarily concentrated in cluster I (Pandita et al. 2023), which shows that the genetic relatedness (as determined by SSR marker-based profiling) strongly correlates with nutritional quality of rice grains. Furthermore, the study identified a number of high nutritive value genotypes. The genotypes that showed more cumulative mineral uptake are K-44 (Fe, K, Mg and Na), HA-8 (Ca, Zn, Na, P), HA-4 (P, K, Ca), K-16 (P, Fe, K), K-19 (Fe, K, Ca), K-30 (Fe, Mg, Ca, Zn), K-31 (P, Fe, Na), K-34 (Fe, K, Ca) and the genotypes that demonstrated the most cumulative nutrient content are HA-4 (Protein, Starch and Amylose), K-47 and K-40 (protein and amylose), K-45 and K-41 (amylose and crude fibre), HA-6 (protein and amylopectin), K-42 (amylopectin and crude fibre), K-37 (amylopectin and crude fibre), K-23 (starch and amylose), K-27 and HA-10 (starch and crude fibre), K-13 and K-30 (protein and amylopectin), K-24 (starch and amylose). All these genotypes can be utilized in the smart breeding of high-nutrient-value rice varieties or some can be directly commercialized. These can assist breeders in identifying suitable parents for crossing and reorient research towards the development of 'quality' rice varieties for biofortification.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12892-024-00260-5.

Acknowledgements The corresponding author is thankful to Department of Biotechnology, Government of India for the financial support in the form of research grant.

Author contributions AMH conceptualized, designed, guided and provided facilities for research; DP performed research; NRS provided the rice germplasm; DP, IAS and MAM performed analysis; DP, ART and AMH wrote the article with input from all authors. All authors contributed to the article and approved the submitted version.

Funding AMH received funding from Department of Biotechnology, Government of India in the form of research grant.

Data availability It will be made available on request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest and nothing to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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Article *in* International Journal of Advanced Biochemistry Research · November 2024

DOI: 10.33545/26174693.2024.v8.i10Ss.2764

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International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(10): 1542-1547
www.biochemjournal.com
Received: 14-08-2024
Accepted: 13-09-2024

A study on seed maturity indices of *Ulmus wallichiana* Planchon in Kashmir

Mehak Aslam, Vaishnu Dutt, KN Qaisar, Amjad M Husaini, AA Mir, BA Bhat, PA Khan, GM Bhat and RA Mir

Mehak Aslam
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

Vaishnu Dutt
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

KN Qaisar
Controller of Examination Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Srinagar, Jammu and Kashmir, India

Amjad M Husaini
Genome Engineering and Societal Biotechnology Laboratory, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Srinagar, Jammu and Kashmir, India

AA Mir
Division of Forest Biology and Tree Improvement, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

BA Bhat
Division of Agri Econ. & Statistics, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Wadura, Jammu and Kashmir, India

PA Khan
Division of Forest Biology and Tree Improvement, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

GM Bhat
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

RA Mir
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

AA Mir
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

Corresponding Author:
Mehak Aslam
Division of Silviculture and Agroforestry, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Benhama, Ganderbal, Jammu and Kashmir, India

DOI: https://doi.org/10.33545/26174693.2024.v8.i10Ss.2764

Abstract

Ulmus wallichiana Planchon (Himalayan elm), a tree species found in the mountainous regions, is known for its multifarious uses. However, overexploitation and problems in its propagation has resulted in it currently reaching the stage of a vulnerable plant species. The present investigation entitled a study on seed maturity indices of *Ulmus wallichiana* Planchon in Kashmir was carried out to determine the optimal time for collection of Himalayan elm seeds. Samaras/seeds were collected from *U. wallichiana* trees, beginning in the fourth week of April followed by subsequent collection every 10 days in the year 2021 and 2022, for analysing maturity indices in relation to various physiological characters of seed. The samara colour of *U. wallichiana* transitioned from cascade green with shining light green wings to pale green colour with light brown centre as maturation progressed, in both years. Moisture content in both years declined from initial to final collection between 88.25 and 86.97% to 10.27 and 8.96 %. Dry matter accumulation steadily increased until the third collection, then rose significantly by the fourth (between 79.91 and 80.14%) and fifth collections (91.73 and 92.04%) in 2021 and 2022. *U. wallichiana* seeds collected at the final harvest showed maximum germination in both years (81.00% and 81.50% in 2021 and 2022 respectively), when seeds had dried. Thus, the exact knowledge of maturity time of Himalayan elm seeds i.e., 1st week of June in Kashmir valley, will be essential to avoid the collection of immature and non-viable seeds so as to synchronize artificial regeneration of this species, whose regeneration is poor in natural habitats.

Keywords: Seed, maturity indices, *Ulmus wallichiana*, vulnerable, physiological characters

Introduction

Plant diversity is crucial for the survival of animal species and serves as a vital source of knowledge and research for humans, including access to bioresources and sustainable practices (Huang, 2011) [17]. However, forests, once believed to be abundant, are rapidly diminishing due to a number of factors. This leads to the extinction or fragmentation of populations into isolated regions (Frankel *et al*., 1995, Heywood and Iriondo, 2003) [10, 16]. *Ulmus* spp., commonly known as elms, are particularly vulnerable to overexploitation and habitat degradation. The Himalayan elm (also known as Kashmir elm or Bhutan elm), scientifically known as *Ulmus wallichiana* Planchon, has suffered significant exploitation, primarily due to extensive lopping for forage and firewood, as well as bark removal for rope production. This has led to irreparable damage to the tree, hindering its natural regeneration. Challenges in afforestation arise from a high percentage of empty seeds and limited viable seed availability. Additionally, difficulties in rooting stem cuttings impede propagation efforts by farmers and enthusiasts. These problems and challenges, have resulted in the Himalayan elm being classified as a vulnerable species.

Giving brief of the species, *U. wallichiana* Planchon thrives in mountainous regions spanning from Afghanistan to Nepal (Jain, 1991) [20], typically found at altitudes of 1800 to 3000 meters in moist ravines and temperate forests alongside various other tree species, generally with *Celtis tetrandra*, *Hippophae salicifolia*, *Juglans regia*, and *Betula alnoides* (Singh and Rawat, 1999; Haq, 2012) [39, 13], in Kashmir. This majestic tree, reaching heights of up to 30 meters, boasts a broad crown and furrowed greyish-brown bark with elliptic-acuminate leaves which serve as fodder. Flowers occur in clusters on the branches with the maximum flowering time being from March to April. The samarae are generally orbicular, less than 13 mm in diameter (Melville and Heybroek, 1971) [26] and seeds are central and hirsute to subglabrous.

The tree yields strong fiber for cordage and ropes from its bark, and its timber is prized for manufacturing and fuel. The young shoots are prized as buffalo fodder and for crafting light boards (Gamble, 1922; Pearson and Brown, 1932) [11, 32]. Beyond its practical uses, *U. wallichiana* Planchon holds medical significance, in traditional bone treatment in the Himalayan region (Arya, 2008; Arya and Agarwal, 2008) [2, 3]. Additionally, extensive studies have revealed that this plant species possesses a wide range of pharmacological properties, which include anti-inflammatory, anti-hypertensive, anti-cancer, antioxidant, antibacterial, antifungal, anti-hyperglycemic, cardioprotective, and neuroprotective effects. (Aslam *et al*., 2024) [4]. Despite its medical significance and practical uses, the Himalayan elm faces endangerment due to exploitation and regeneration difficulties. Classified as vulnerable by IUCN in 1998 and endangered by Walter and Gillett in the same year, urgent conservation efforts are needed.

Plant propagation has long been practiced to meet the needs of animal life, particularly humans, with improving techniques serving as a key approach to protect overexploited species (Hartmann *et al.,* 1997) [15]. Seed propagation remains the primary method of regeneration in silviculture, in both temperate and tropical regions, preserving genetic diversity effectively. Recognizing the stage of seed maturation just before dehiscence is crucial for obtaining high-vigour seeds, as collecting seeds after dehiscence may result in lower quality due to exposure to environmental conditions (Bonner, 1972) [6]. Understanding maturity timing is essential to avoid harvesting immature or non-viable seeds. The reproductive cycle of a plant begins with flower bud production, continues through anthesis, fruit development, and seed storage material accumulation, and culminates in physiological maturity, marked by the seed reaching its maximum dry weight (TeKrony and Egli, 1997) [41]. The period between seed maturity and dispersal can vary due to annual climate fluctuations. Hence, determining the optimal seed collection time involves understanding the relationship between seed maturity, colour, fresh weight, dry weight, and moisture content. Thus, this research study was undertaken to determine the best time for seed collection so as to be able to propagate large numbers of high-quality Himalayan elm plants through seeds more effectively.

Materials and Methods

Three seed-bearing trees of *U. wallichiana*, displaying superior morphological or phenotypical characteristics, were chosen for the study. These trees are located in the forests of Rangnar Braaripathri Ajas, within the Bandipora district of Kashmir. The study was carried out for two years, in 2021 and 2022. Samaras/seeds were collected from these trees at various intervals, beginning in the fourth week of April followed by subsequent collection every 10 days till the first week of June. During the last collection, it was observed that trees had shed majority of their seeds. Following each collection, samples of samaras/seeds were transported to the laboratory at the Faculty of Forestry, SKUAST-Kashmir, located in Benhama Ganderbal. Samples brought from all three trees were combined. During each collection, the colour of the samaras was noted. The weight (in grams) of 1000 samara was measured in four replicates, each consisting of 1000 samara, using a sensitive top pan balance. The growth of samaras was assessed by observing the increase in the fresh weight of 1000 samaras and the percentage of dry matter. Moisture content of the samaras was measured based on their fresh weight after drying at 103±1 °C for 16±1 hours, in accordance with ISTA (1993) guidelines. The germination test was carried out on all collection dates to verify seed maturity. To assess germinability, seeds were placed on top of seed germination paper and incubated at 25°C in a laboratory germinator. The testing period lasted 21 days, with daily documentation of germination progress commencing on the third day after seed placement. Seeds were considered germinated as soon as the radical emerged and were counted accordingly. Analysis of variance (ANOVA) along with the least significant difference (LSD) at a significance level of $p \leq 0.05$ was employed to evaluate the variations in the recorded data concerning the maturity indicators and seed germinability of Himalayan elm.

Results and discussion

Understanding the physical transformations in seed characteristics plays a significant role in determining the indices of seed maturity. Recent research indicates that understanding the physiological maturation process of seeds is crucial for determining the point at which seeds achieve their highest quality and the optimal time for fruit harvesting (Magalhaes *et al*., 2019; Emmanouilidou *et al.,* 2020) [23, 9]. In this study, observations revealed distinct colour transitions in the samaras throughout seed development. Initially, they appeared cascade green with shining light green wings at collection I, followed by a shift to light mint green with shining light green wings on collection III, and ultimately, they attained a pale green colour with light brown centre and parched wings at the final harvest (collection V) in the first week of June, observed in year 2021 and 2022. As maturation progressed, the samaras of *U. wallichiana* transitioned from cascade green to light brown, indicating a significant change in colouration. In broad-leaved species, alteration in fruit/seed colour serves as a valuable indicator of maturation. Alike colour changes during maturity have been observed in other plant species such as *Gmelina arborea* (Saralch and Singh, 2013) [36], *Adina cordifolia* (Jeena *et al.*, 2012) [21], *Acer caesium* (Phartyal and Thapliyal, 2006) [33], *Carrisa opaca* (Mithani *et al*., 1987a) [27], *and Ficus benjamina (*Mithani *et al.*, 1987b) [28]. Additionally, colour change has been suggested as a maturity indicator in *Azadirachta indica* (Bharathi *et al*., 1996) [5], *Quercus* and *Prunus cerasoides* (Tewari *et al.*, 2011) [43], as well as in *Bauhinia retusa* (Upadhayay *et al*., 2006) [44]. In their study on the maturity indices of *Aesculus indica* Colebr. seeds, Majeed *et al.* (2010) [24] observed that the seeds displayed a shiny chocolate brown color upon reaching maturity.

The data indicates significant differences in the fresh weight of samaras across the collection dates in both years (2021 and 2022) of the study. During initial collections, there was an increase from 25.6 g and 23.4 g in the year 2021 and 2022 respectively at collection I to 32.4 g and 33.5 g in 2021 and 2022 respectively at collection II (Table 1). This difference was significant and was followed by a sharp decline in fresh weight of samara. The minimum fresh weight of 4.3g and 4.1g in year 2021 and 2022 respectively, was observed at final collection (Table 1). Fresh weight of *U. wallichiana* seeds increased upto the mid-point of seed development and thereafter declined till the time of full maturity, as also reported in *Acer caesium* (Phartyal and Thapliyal, 2006) [33]. Data in Table 1 depicts a significant decline in moisture content in both years, from initial to final collection i.e., 88.25 to 10.27 % during year 2021 as well as from 86.97 to 8.96% during year 2022 (Fig. 1). Decline was gradual upto the collection III and then there was a sharp fall in the last two collections in both years. (Table 1). However, moisture content recorded at all collection dates differed significantly. Moisture content has been used as a reliable maturity indicator by numerous researchers. Decline in moisture content is closely related to seed development and maturity (Adams and Rinni, 1981; Shah, 2005; Pandit *et al.*, 2002; Shah *et al.*, 2006) [1, 37, 31, 38], as observed in *U. wallichiana*. The maximum germination percentage in *U. wallichiana* occurred when the moisture content of seed was in the lower range between 7 and 8% (Fig. 1). Similar results were obtained in *Pinus roxburghii* in which maximum germination occurred when the seed moisture content was low 21% in Yr1 and 22% Yr2 (Mittal *et al.*, 2020) [29]. At physiological maturity, all nutrients cease to flow into fruit from parent plant and thus facilitate the drying of fruits and seed to low moisture content without losing viability (Harrington, 1972) [14]. Seeds of other forest tree species are considered mature below a critical level, for example, 30-40% in *Ulmus pumila* (Grover *et al.*, 1963) [12], 13% in *Dalbergia sissoo* (Joshi, 2000) [22], 44% in *Azadirachta indica* (Nayal *et al.*, 2000) [30], 60% in *Populas ciliata* (Pandit *et al.*, 2002) [31] and 14% in *Acer caesium* (Phartyal and Thapliyal, 2006) [33]. Tewari *et al.* (2016) [42] observed that the seed moisture content decreased from 87% to 15.6% in *Mallotus phillipensis*, as the collection progressed. Moisture content in developing seed is related to protein synthesis and Rosenberg and Rinne (1986) have shown that seed moisture content must drop below 60% in order to trigger protein synthesis. Without this happening, seedling growth is arrested.

Dry matter accumulation increased gradually and consistently until the third collection (27.06% in year 2021 and 26.29% in year 2022), followed by a significant rise between the fourth (79.91% in year 2021 and 80.14% in year 2022) and fifth collections (91.73% in year 2021 and 92.04% in year 2022) (Table 2). Developing seeds begin to gain weight through the accumulation of nutrients and water uptake. Initially, seed filling occurs slowly as cell division and elongation take place. Subsequently, dry mass accumulation accelerates until the seeds reach their maximum dry weight (Fig. 1). A similar pattern of dry matter accumulation has been observed in several other species, including *Fraxinus pennsylvanica* (Bonner, 1973) [8], *Quercus shumardii* and *Q. alba* (Bonner, 1974) [7] and *Quercus douglasii* (McCreeary and Koukoura, 1990) [25]. Seeds harvested at the collection I failed to germinate in both years, but on and after collection II, germination of seeds collected began (Table 2). Data in Table 2 revealed that only 3.50% and 4.00% germination was recorded for year 2021 and 2022 respectively, for seeds harvested on collection II. Germination of seeds increased progressively till the final harvest with a maximum germination of 81.00% and 81.50% in year 2021 and 2022, respectively (Table 2). The germination of *U. wallichiana* seed was at its maximum at the final harvest (Fig. 1) when seeds had dried, indicating that physiological maturity was reached around this time. The stimulation of germination by drying appears to be universal, since even in recalcitrant seed, a small reduction in moisture content has this effect (Suszka, 1966; Probert and Brierley, 1989) [40, 34]. Thus, desiccation plays a vital role in seed germination, as it might be necessary pause essential for a switch over from developmental process to those essential for germination. Similar results were reported by Mittal *et al.* (2020) [29] who reported a low germination% in *Pinus roxbhurgii* during the first collection, which gradually increased with each collection date with the maximum being observed at the final collection (81.3±1.45% in Yr1 and 79.0.0±1.7% in Yr2). In *Adina cordifolia*, germination increased with the advancement of collection date with the maximum germination percentage being recorded to be 84.03%. (Jeena *et al.*, 2012) [21]. Phartyal and Thapiyal (2006) [33] also reported that germination in *Acer caesium* showed a highly significant correlation with dry matter and moisture content during maturation.

Table 1: Variation in fresh weight (g) and moisture content (%) of Himalayan elm (*Ulmus wallichiana* Planchon) on different collection dates

Collection number	Date of collection	Fresh weight (1000 seeds) (g)			Moisture content (%)		
		Year 1 (2021)	Year 2 (2022)	Pooled mean	Year 1 (2021)	Year 2 (2022)	Pooled mean
I	25-04-2022	25.6	23.4	24.5	88.25 (69.95)	86.97 (68.84)	87.61 (69.39)
II	06-05-2022	32.4	33.5	32.9	82.85 (65.53)	82.93 (65.59)	82.89 (65.56)
III	16-05-2022	12.2	13.4	12.8	72.94 (58.65)	73.70 (59.15)	73.32 (58.90)
IV	26-05-2022	7.6	8.0	7.8	20.09 (26.63)	19.85 (26.46)	19.97 (26.54)
V	05-06-2022	4.3	4.1	4.2	10.27 (17.71)	8.96 (16.79)	8.11 (16.55)
CD ($p \leq 0.05$)		0.31	0.36	0.35	1.53	1.20	1.39

Values in the parenthesis are arcsine transformed values

Table 2: Variation in dry matter (%) and germination (%) of seeds of Himalayan elm (*Ulmus wallichiana* Planchon) collected on different collection dates

Collection number	**Date of collection**	**Dry matter (%)**			**Germination (%)**		
		Year 1 (2021)	**Year 2 (2022)**	**Pooled mean**	**Year 1 (2021)**	**Year 2 (2022)**	**Pooled mean**
I	25-04-2022	11.76 (20.05)	13.03 (21.16)	12.40 (20.61)	0.00 (0.01)	0.00 (0.01)	0.00 (0.01)
II	06-05-2022	17.15 (24.47)	17.07 (24.41)	17.11 (24.44)	3.50 (10.78)	4.00 (11.54)	3.75 (11.16)
III	16-05-2022	27.06 (31.35)	26.29 (30.85)	26.68 (31.10)	22.50 (28.32)	22.00 (27.97)	22.25 (28.14)
IV	26-05-2022	79.91 (63.37)	80.14 (63.54)	80.03 (63.46)	51.00 (45.57)	51.25 (45.72)	51.13 (45.64)
V	05-06-2022	91.73 (73.29)	92.04 (73.61)	91.89 (73.45)	81.00 (65.21)	81.50 (65.62)	81.25 (65.42)
CD ($p \leq 0.05$)		1.54	1.18	1.38	2.61	3.40	3.03

Values in the parenthesis are arcsine transformed values

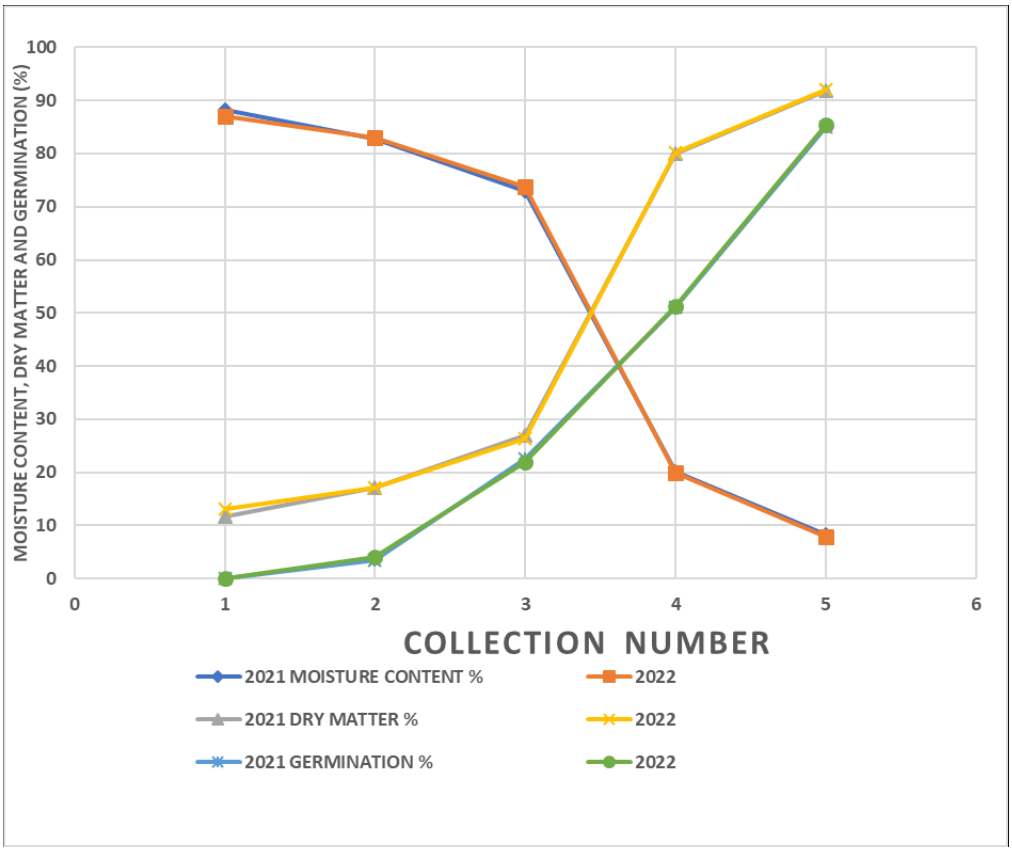

Fig 1: Chronological changes with maturity in moisture content, dry matter and germination percentage of Himalayan elm (*U. wallichiana* Planchon) seeds in year 2021 and 2022.

Conclusion

To synchronize artificial regeneration of *Ulmus wallichiana*, whose regeneration is poor in natural habitats, exact knowledge of maturity time will be essential to avoid the collection of immature and non-viable seeds. This study conducted on maturity indices revealed that the optimal time for collection of Himalayan elm seeds, when a large number are viable and germinable is the 1st week of June in Kashmir valley.

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