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Nutritional profiling of pigmented and scented rice genotypes of Kashmir Himalayas

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Abstract
Assessment of nutritional diversity in a crop germplasm is vital. In the present study a set of 19 rice accessions, including pigmented, scented landraces, Basmati-type genotypes and popular varieties of Northern Himalayan region were evaluated biochemically for their nutritional value. De-husked rice grains were assessed for seven major parameters. A highly aromatic landrace of Kashmir Himalayas, which is grown in mid altitude cold regions, showed maximum total protein (8.86%) content as well as highest fiber content (3.31%). However, it recorded the lowest starch content of 70.45% while a popular high yielding variety grown in the plains of Kashmir valley 'Shalimar rice-1' recorded the highest (79.36%) starch content as well as maximum amylose percentage (24.34%). Total Phenol content showed a wide range from 4.87 to 1.02 mg/g, with maximum in a pigmented rice genotype 'Purple rice' while lowest in Jhelum, a popular high yielding rice variety. Besides, purple rice also had maximum total anthocyanin (9995.34 µg/g) content, while lowest (5943.14 µg/g) was recorded in Jhelum. Similarly, total carotenoids too varied in a wide range, with 'Khuch' recording almost 10 times (0.022 µg/g) than the lowest (0.002 µg/g) in Shalimar Rice-1 and Jhelum. These results indicate that scented and pigmented rice genotypes of Kashmir Himalayan region are of better nutritional quality than the conventional high yielding varieties and could be promoted as 'Specialty' rice for better economic returns to the farmers.

Keywords: Pigmented rice, Scented rice, Protein, Starch, Fiber, Amylose, Carotenoids, anthocyanin, phenols, Biochemical properties, Characterization

1. Introduction
In order to safeguard the livelihood security of rice farmers and nutritional security of population, it is important to conduct research oriented towards increasing farmer's 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 with this objective, but these biofortified products are not affordable for common man. 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 rest of India, especially with respect to cold tolerance [1]. Further, Himalayan region of Kashmir grows many '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) [2]. In spite of this, most of the rice breeding programmes in the region have remained focused only on developing resistance to disease, cold or high yields, while nutritive quality and consumer acceptability has been largely ignored [1, 3, 4].
Under changing climatic conditions there is already an adverse impact on some of these conventionally bread high yielding varieties of rice, best suited for high input agriculture. Environmental stresses (low temperature and water scarcity) coupled with cultivation under marginal conditions by resource poor farmers cause adverse impact on the qualitative and quantitative characteristics of these rice genotypes, especially due to physiological imbalance in mineral uptake. Hence, identification and revival of 'indigenous high-value' rice genotypes have assumed special importance under the present scenario [3]. We have recently characterized some of these high-value genotypes using SSR markers and showed that these genotypes are genetically diverse [5]. Amongst these cold tolerant lines are several local pigmented rice genotypes and aromatic landraces, which used to be popular until the arrival of Green Revolution based High Yielding Varieties. However, due to crop diversification and diversion of land towards more remunerative horticultural crops and high yielding varieties, erosion of rice biological diversity took place.

Government of India has recently set up a target of doubling farmers' income by the year 2022. Rice is grown in Kashmir on low lands and under irrigated conditions, which is not generally suitable for plantation of horticultural crops because of high water table. Rice farmers are at a disadvantage in comparison with Apple growers' in terms of economic returns. As such, there is an urgent need to make rice farming more remunerative and one way of achieving it would be to identify and promote 'high-value rice genotypes'. These genotypes can be either used directly for commercialization or help breeders to identify suitable parents for crossing and re-orient research towards development of 'quality' rice varieties for biofortification [2]. The present study was conducted with the prime objective of evaluating the genetic/nutritional value of these cold-tolerant genotypes, which was hitherto unknown. More importantly, the genotypes identified in the present study can be revived and popularized for cultivation, because of their inherent potential to address the issues of malnutrition in the poor segments of the society.

2. Materials & methods

Many indigenous rice lines were collected from different agro-ecological regions of Jammu and Kashmir state, India, and maintained at High Altitude Research Station, Larnoo (2400 m amsl) and Mountain Research Centre for Field Crops (MRCFC), Khudwani (1650 m amsl). The basic material for the present study consisted of nineteen diverse genotypes of rice (*Oryza sativa* L.) (Table 1). Leaf as well as grain was selected from the germplasm collection maintained at Mountain Research Centre for Field Crops (MRCFC) Khudwani SKUAST (K) Anantnag. The research station is located in south Kashmir 5 km away from Anantnag town. The location is 1680m a.m.s.1 and situated 34½ ˚N of latitude and 77.0 ˚E of longitude. The grain samples were selected on the basis of morphological variability and were de husked manually using pestle mortar. The selected rice accessions used were Grey Rice, Zag 2, Zag 3, Zag 4, Zag 7, Zag 10, Zag 11, Zag 13, Zag 14 (8 selections of landrace Zag from different locations), Zager, Kawa-kreed, Loual anzul, Khuch, Purple rice, Mushk budji, Kamad, Pusa Sugandh-3, Jhelum and Shalimar rice 1.

2.1 Total protein (%)

Micro-Kjeldal method was used to determine nitrogen content [6]. Half gram of dehusked rice samples in powdered form per replicate was placed in Kjeldal tubes and 5 g of digestion mixture (potassium sulphate + iron sulphate + copper sulphate in the ratio of 5: 0.5: 0.25) was added. After adding 10 ml of concentrated H_2SO_4, the mixture was heated till colour changed to green. Then the tubes were cooled and 10 ml of distilled water was added to each sample. Then tubes were fitted in assembly. Mixture from tubes was dipped in flask fitted in assembly containing 25 ml of boric acid (4.0%) and 5 ml mixed indicator (2 parts of 0.2% methyl red in ethanol with 1 part of 0.2% methylene blue in ethanol). Then 40-50 ml of NaOH (40.0%) was added to it till colour changed to brown. Flask containing boric acid and indicator was titrated with 0.1 N HCl till changed to brown.

Protein estimation was calculated from nitrogen content (N%) in accordance with the following formula:

$$N\% = \frac{\text{Quantity of sample- Blank (ml)} \times \text{Normality of HCl} \times 14}{1000 \times \text{Weight of Sample (g)}} \times 100$$

Protein (%) = N% × 5.95
where, 5.95 is conversion factor for rice

2.2. Starch (%)

Hodge and Hofreiter (1962) [7] method was used Conversion of starch to sugars was done by addition of 100 ml water and 30 ml concentrated hydrochloric acid to 1.0 g sample and boiling in water bath for 2.5 hours.To this solution The solution was cooled, neutralized and made to and made to 250 ml volume. After precipitating proteins and heavy metals using 45 per cent lead acetate and potassium oxalate, the contents were filtered through Whatman No. 41 filter paper. Five ml each of fehlings solution (A) and (B) were taken in a titration flask containing 25 ml of distilled water and titrated against solution (I) for estimation of total sugars, till red colour was observed. Then, 2 drops of methylene blue (indicator) were added and titration was continued till brick red precipitate was observed. During the entire period, the flask was kept on burner to keep the contents hot. Percentage of total sugars was calculated by using the formula:

$$\text{Total sugars} = \frac{\text{Fehlings solution equivalent} \times \text{volume made}}{\text{Ml of filtrate used} \times \text{sample weight}} \times 100$$

Starch = (total sugars – reducing sugars) × 0.9

2.3. Amylose (%)

Total amylose of the milled rice grain was determined by method adopted by [8]. To 100mg of powdered sample 1ml of distilled ethanol was added after mixing it well, 10 ml of 1 N NaOH was added and was left overnight and volume was adjusted up to 100ml. 2.5 ml of extract was taken and 20 ml of water was added in addition to 2-3 drops of phenolphthalein indicator. 0.1 N HCl was added drop by drop until the pink colour disappears. Then 1 ml of Iodine reagent was added and the volume was raised up to 5 ml and the absorbance was recorded at 590nm.

2.4. Crude fibre (%)

Crude fibre was estimated using standard method of Maynard [9]. Two grams moisture free de husked rice was transferred to the spoutless beaker and 200 ml of 1.25 per cent H2SO4 was added. Beaker was placed on digestion apparatus with pre-adjusted hot plate and boiled for 30 minutes. The beaker was then removed from the hot plate and filtered through muslin cloth. Washed the residue with hot water till it was free from acid. Transferred the material into the same beaker with 200 ml of 1.25 per cent NaOH. Again, refluxed the contents for 30 minutes. The contents were transferred to sintered glass crucible and washed with boiling water and alcohol thrice and dried at 1000C in hot air oven. After cooling in desiccator, the weight of crucible with residue was recorded. The crucible was transferred to muffle furnace at 550±50C for 2-3 hours, cooled and weighed again. Crude fibre content was calculated from loss in weight of crucible on incineration in muffle furnace.

2.5 Phenols (mg/g)

Total phenolic content in milled rice grain was estimated by method of [10]. 1g of dehusked grain sample was grinded in 10 time volume of 80% ethanol and then centrifuge at 10,000 rpm for 20 min. Supernatant was preserved and was re extracted with 5 times vol. of 80% ethanol, and the supernatant was dried. The residue was dissolved in known

vol. of distilled water, and 2ml of aliquots were pipittet out, and volume was raised to the vol. of 3ml. 0.5ml of folin-ciocalteu reagent was added. After 3 minutes 2ml of 20% sodium carbonate was added and placed in boiling water for 1 minute and then cooled. Absorbance was measured at 650nm against a blank. The different concentrations of Catechol was used for standard curve preparation and from that standard curve, the concentration of rice samples was calculated.

2.6 Carotenoids (µg/g)

Carotenoids were estimated in rice grain by method adopted by [11]. A known weight (1g) of homogenized rice sample was taken and subjected to centrifugation. Supernatant was discarded and pellet was washed 2-3 times with distilled water. To the pallet 2-3 ml of acetone (85%) was added and was again centrifuged and supernatant was collected. Extraction was repeated with acetone till the supernatant becomes colorless. All the fractions of supernatant was collected to make up the final known volume to read the absorbance at 450 nm using 85% acetone as a blank.

$$\text{Carotene} = \frac{D \times V \times 10}{2500}$$

Where, D is Absorbance at 450nm; V is Vol. of the extract, and F is Dilution factor.

It is assumed that the pigments had an average extinction coefficient of 2500.

2.7 Total anthocyanin (mg/100 g)

Total anthocyanin content in milled rice was determined by method as adopted by [12]. 1 gm of grain sample was crushed in alcohol and was filtered, followed by centrifugation and the extract was collected. Out of this whole 1 ml of extract was pipetted out and 3ml of HCL was added in aqueous methanol. 1 ml of anthocyanin reagent was added to it, followed by incubation for 15 minutes in dark. Absorbance was measured at 525 nm with 95% ethanol as a blank. The amount of anthocyanin in the sample was calculated from standard curve prepared by cyanin hydrochloride.

3. Result and Discussion

3.1 Total protein (%)

Protein content is significantly influenced by variety, environment, season, and nitrogen fertilization [13]. In the present study the total protein content was found highest in scented landrace Mushk budji (8.860%) (Table 2). The minimum protein content was found in Khuch (5.510%) followed by Purple rice (5.73%). The protein content in Jehlum and Shalimar rice-1 was recorded as 6.55 and 8.57%, while in a previous study by [14] it was found to be 8.42 and 8.65% respectively. In the present study, Pusa sugandh-3 showed higher total protein (8.263%) than that of the pigmented rice genotypes, and is similar to that reported in Pusa sugandh-2 (8.2%) [15]. The protein content of basmati types reported earlier ranges between (7.75 to 8.96%) in Pusa basmati-1 and Tarori basmati [15]. Similar study was conducted by [16] on six different rice varieties marketed in Penang, Malaysia (locally grown and imported) and found that protein content of all the varieties evaluated ranged between 5.96 to 8.16%, with protein content in Pakistani Basmati as 7.75% [16]. A high protein content of 9.84% was reported for Basmati-370 by [17]. Protein content more than 10% is considered as 'high' [18]. The total protein content ranged from 6.63-8.46g/100g in eight pigmented rice genotypes grown in southern Thailand [19]. High protein content (9.52%) has been reported in Njavara rice, a medicinal rice variety, which was 16.5% higher than non medicinal rice Jyoti (7.97g/100g) and IR 64 (7.95 g/100g) [20].

3.2 Starch (%)

It is the major dietary source of carbohydrate and is the most abundant storage polysaccharide in plants [21]. It is a complex carbohydrate source and often simply called starches and tend to be high in fiber. Highest starch content was recorded in Shalimar rice-1 (79.30%) followed by Jhelum (78.89%), while the lowest in Mushk budji (70.45%) and Pusa sugandh-3 (71.32%) (Table 2). In a study on ten aromatic long and short grained varieties the starch content ranged from 64.6 to 89.1% in Pusa sugandh-2 and Kalanamak, respectively. In Pusa Basmati-1 it was 70.1% and in Toroari Basmati 78.2% [15]. The starch content as reported by [14] in Shalimar rice-1 (69.76%) and Jhelum (70.26%) is lower than that reported in the present study, which may be due to difference in the estimation methods of [7] (present study) and [22] or due to the difference in soil nutrients.

3.3 Amylose (%)

Amylose content is one of the most important determinants of rice quality. Rice contains both soluble and insoluble amylose. Insoluble amylose of rice directly affects kernel firmness and inversely affects stickiness and glossiness of cooked grain. Amylose content ranged from 18.23 to 24.43%, with minimum in Zag-10 and maximum in Shalimar rice-1 (Table 2). Shalimar rice-1, being a white rice variety, has the higher level of amylose content and therefore can show retrogradation. On the contrary, Zag-10 showing least amylose content will not be prone to retrogradation.

Mushk budji had amylose content of 24.12%. Pusa sugandh-3 had 23.56% amylose content, which is similar to that reported earlier 26.78% [23]. Rosniyana *et al.* (1995) have reported amylose content of 19.9% in Basmati-370, a thin long aromatic rice variety similar to Pusa sugandh-3. The amylose content reported in an earlier study [15] on rice varieties varied from 16.8% (CMR 839) to 25.05% (Geetanjali). In a similar study, [20] has reported amylose content of 22.7% (Njavara), 22.9% (Jyothi) and 24.3% (IR-64). This variation in amylose content may be due to variation in the temperature during grain ripening stage, whereby the amylase content generally decreases as the mean temperature increases [18]. In addition, the amylose content is also influenced by nitrogen fertilization, whereby the value decreases slightly with nitrogen fertilization but is not effected by the stage at which nitrogen is applied [24].

3.4 Fiber (%)

Fiber content is very less in rice as the maximum amount of fiber is present in the husk, which is removed during the process of milling and leads to loss of crude fiber, and therefore milled rice is poor in fiber content. Highest fiber content was found in Mushk budji (3.31%) and lowest in Zager (1.38%) (Table 2). In a similar study by [19] the fiber content ranged from 0.16-0.35g/100g amongst the eight varieties of pigmented rice grown in southern Thailand. Rosniyana *et al.* (1995) have reported fibre content of 0.29% in Basmati-370, while in the present study the fibre content of long-grain aromatic variety Pusa sugandh-3 was 2.94%. Thomas *et al.* (2013) 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%. Similar, Deepa *et al.* (2008) observed that the total dietry fiber content in Njavara was found as 8.08%, which was 34 to 44% higher than Jyoti (5.82%) and IR-64 (4.96%). These results show that there is a huge variation in the fibre content amongst the rice genotypes of different geographical regions.

3.5 Phenols

Maximum phenol content was recorded in pigmented rice varieties such as Purple rice (4.87 mg/g), Khuch (3.34 mg/g), Kaw kreed (2.24 mg/g) and minimum in Jhelum (1.02 mg/g) and Zag-3 (1.21 mg/g) (Table 3). There is linear correlation between phenolics and pigments, and therefore purple rice has comparatively higher phenolic content. The phenolic compounds are mainly associated with the pericarp in rice. Gorinstein *et al.* (2007) [25] compared the total polyphenols in buckwheat (0.912 mg/g), soybean (0.690 mg/g), amaranth (0.405 mg/g) and Jasmine rice (0.330 mg/g), while in rice bran it was 0.92 mg/g. Besides, grains with darker pericarp colour, such as red and Black rice contain higher amounts of polyphenols [26, 27].

3.6 Carotenoids

Carotenoids are plant pigments that function as antioxidants, hormone precursors, colourants and essential components of the photosynthetic apparatus. β-carotene is a precursor of vitamin A [28]. Maximum carotenoid content was observed in Khuch (0.022 µg/g) followed by Purple rice (0.019 µg/g) and minimum in Jhelum and Shalimar rice-1 (0.002 µg/g) (Table 3). Khuch is known to have highest carotenoid content which is directly attributed with red brown pigments present in it. However, shalimar rice 1 and Jehlum which are non pigmented white rice varieties have lowest carotenoid content.

3.7 Anthocyanin

Anthocyanins are plant-derived polyphenol that produces the red, purple, blue and black pigmentation. Coloured rice contains more anthocyanin than non-coloured rice. Anthocyanins, are recognized as health-promoting food ingredients due to their antioxidant activity [29-31], anticancer [32], hypoglycemic [33], and anti-inflammatory effects [34]. Pigmented rice is a good source of fiber, minerals, and several important amino acids [35], and there is increased interest in these alternative sources of anthocyanins due to a rising demand for economical sources of natural and stable pigments [36].

In the present study maximum anthocyanin content was observed in pigmented rice variety Purple rice (9995.34 µg/g) while minimum in Jhelum (5943.14 µg/g) and Shalimar rice-1 (5962.65 µg/g) (Table 3). Dark purple colour group had higher phenol and anthocyanin content than red, brown, and white colour groups. Gorinstein *et al.* (2007) while studying some selected cereals and pseudocereals observed that highest anthocyanin was in Rice bran 132.0 mg/100 g as compared to the Buck wheat (111.3 mg/100 g), soyabean (100.2 mg/100 g), and Jasmine rice (83.0 mg/100 g). Abdel-Aaal *et al.* (2006) [37] estimated anthocyanin pigments using their modified protocol (Abdel-Aaal *et al.,* 2003) [38] for a wide variety of edible and ornamental black, blue, pink, purple-red and white wheat barley, corn, rice and wild rice and quantified their potential as a natural colorant or functional food ingredient. The total anthocyanin varied from 27 µg/g (wild rice) to 3276 µg/g (black rice), while red rice recorded 93.5 µg/g. Yodmanee *et al.* (2011) [19] worked on eight varieties of pigmented rice in southern Thailand in which the highest anthocyanin content was found in BWR-96044 (2453.6 µg/g) and least was found in KN (97.9 µg/g).

Table 1: List of the rice genotypes used in the present study.

S. No.	Genotype	Description	S. No.	Genotypes	Description
1.	Grey rice	Long and medium bold grown at 1600-1650 mamsl (introduced from Turkey)	11.	Pusa sugandh-3	Scented Basmati type grown at 1540-1600 mamsl; introduced variety grown in Budgam, Anantnag and Kulgam areas
2.	Zag 2	Japonica type short and bold red rice; land race grown in Uri at 1850-2200 mamsl	12.	Khuch	Japonica type short and bold red rice; land race grown in Larnoo at 1850-2200 mamsl
3.	Bahrigu Dhan (Zag 3)	Japonica type short and bold red rice; land race grown in Malang, Himachal Pradesh at 1850-2200 mamsl	13.	Purple rice	Long and medium bold purple colour grain; grown at 1600-1650 mamsl (introduced but origin unknown)
4.	Kund Zag (Zag 4)	Japonica type short and bold red rice; land race grown at 1850-2200 mamsl	14.	Kamad	Japonica type scented short and bold off-white; land race grown at 1850-2200 mamsl
5.	Zag 7	Japonica type short and bold red rice; land race grown at 1850-2200 mamsl	15.	Kawa kreed	Japonica type short offwhite; land race grown at 1850-2200 mamsl
6.	Zag 10	Japonica type short and bold red rice; land race grown in Larnoo at 1850-2200 mamsl	16.	Loual anzul	Japonica type short offwhite; land race grown at 1850-2200 mamsl
7.	Zag 11	Japonica type short and bold red rice; land race grown at 1850-2200 mamsl	17.	Jhelum	Indica type medium bold grain; variety released in 1996 and grown at 1540-1650 mamsl
8.	Cheolweon (Zag 13)	Japonica type short and bold red rice; introduction from Korea and grown at 1650 mamsl at Khudwani	18.	Mushk budji	Japonica type scented short and bold off-white; land race grown in Larnoo etc at 1850-2200 mamsl
9.	Zag 14	Japonica type short and bold red rice; land race grown at 1850-2200 mamsl	19.	Shalimar Rice-1	Indica type medium bold grain; variety released in 1996 and grown in Valley at 1540-1600 mamsl
10.	Zager	Japonica type short offwhite; land race grown at 1850-2200 mamsl		-	-

Fig 1: Grain and the de-husked rice genotypes: a) Grey rice, b) Zag-2, c) Zag-3, d) Zag-4, e) Zag-7, f) Zag-10, g) Zager, h) Zag-13, i) Zag-14, j) Pusa Sughand-3, k) Purple Rice, l) Khuch, m) Mushk Budji, n) Kawa-Kreed, o) Kamad, p) Loul-Anzul, q) Jehlum, r) Shalimar Rice-1.

Table 2: Nutritional content of the nineteen rice genotypes (milled rice)

Rice genotype	Protein (%)	Starch (%)	Amylose (%)	Fiber (%)
Grey rice	6.170±0.012 (2.58±0.08)	75.210±0.009 (8.70±0.0005)	19.210±0.012 (4.43±0.0010)	1.760±0.012 (1.49±0.004)
Zag-2	5.940±0.010 (2.53±0.06)	77.090±0.012 (8.80±0.006)	19.770±0.010 (4.50±0.0021)	1.523±0.009 (1.41±0.009)
Zag-3	6.380±0.260 (2.62±0.07)	77.440±0.012 (8.82±0.006)	20.110±0.011 (4.53±o.0021)	1.640±0.013 (1.45±0.003)
Zag-4	6.153±0.077 (2.57±0.210	78.560±0.013 (8.89±0.008)	21.223±0.011 (4.66±0.0031)	1.430±0.011 (1.38±0.002)
Zag-7	6.283±0.015 (2.60±0.09)	74.370±0.007 (8.65±0.004)	19.110±0.010 (4.42±0.0012)	2.460±0.010 (1.71±0.005)
Zag-10	6.310±0.012 (2.60±0.08)	73.210±0.008 (8.58±0.002)	18.230±0.009 (4.34±0.0011)	2.550±0.011 (1.74±0.006)
Zag-11	7.040±0.014 (2.74±0.011)	76.890±0.011 (8.79±0.001)	19.760±0.008 (4.50±0.0020)	2.610±0.011 (1.75±0.005)
Zager	6.710±0.012 (2.68±0.10)	74.570±0.010 (8.66±0.003)	19.323±0.009 (4.45±0.0010)	1.383±0.009 (1.36±0.007)
Zag-13	6.970±0.011 (2.73±0.09)	77.670±0.014 8.84±0.006)	21.110±0.013 (4.64±0.0030)	3.210±0.015 (1.92±0.008)
Zag-14	6.430±0.010 (2.63±0.08)	75.330±0.011 (8.70±0.004)	19.783±0.009 (4.50±0.0020)	1.790±0.008 (1.50±0.006)
Khuch	5.510±0.009 (2.45±0.08)	74.223±0.011 (8.64±0.007)	22.330±0.014 (4.77±0.0035)	1.930±0.009 (1.55±0.006)
Purple rice	5.730±0.018 (2.49±0.08)	76.350±0.014 (8.76±0.005)	23.340±0.016 (4.88±0.0038)	1.870±0.006 (1.53±0.006)
Kawa-kreed	5.710±0.310 (2.49±0.08)	76.450±0.014 (8.77±0.003)	22.350±0.013 (4.78±0.0034)	2.270±0.011 (1.65±0.007)
Loual-anzul	5.983±0.015 (2.54±0.42)	75.890±0.013 (8.74±0.004)	23.450±0.014 (4.89±0.0039)	1.990±0.008 (1.57±0.004)
Mushk budji	8.860±0.019 (3.05±0.09)	70.450±0.009 (8.42±0.006)	24.120±0.017 (4.96±0.0041)	3.310±0.019 (1.94±0.011)
Pusa sugandh-3	8.263±0.012 (2.96±0.08)	71.320±0.011 (8.47±0.005)	23.560±0.013 (4.90±0.0040)	2.940±0.014 (1.84±0.009)
Kamad	7.950±0.009 (2.90±0.07)	72.340±0.012 (8.53±0.003)	22.770±0.015 (4.82±0.0033)	1.823±0.009 (1.51±0.006)

Jhelum	6.553±0.007 (2.65±0.10)	78.890±0.014 (8.91±0.009)	23.980±0.019 (4.94±0.0042)	1.850±0.009 (1.52±0.005)
Shalimar rice-1	8.570±0.014 (3.01±0.08)	79.360±0.015 (8.93±0.009)	24.340±0.18 (4.98±0.0045)	1.910±0.005 (1.54±0.004)
Mean	6.74 (2.59)	75.55 (8.69)	21.46 (4.63)	2.11 (1.45)
CD	0.58	0.18	0.28	1.45
SE(d)	0.41	0.12	0.19	0.12

Figures in parenthesis are square root transformed values

Table 3: Total phenol, caroteniods, anthocyanin in nineteen rice genotypes

Rice Genotype	Phenols (mg/g)	Carotenoids (µg/g)	Anthocyanin (µg/g)
Grey rice	1.310±0.011	0.007±.009	8973.230±1.008
Zag-2	1.230±0.012	0.005±.008	8749.620±1.711
Zag-3	1.210±0.011	0.004±.007	8899.977±0.974
Zag-4	1.310±0.017	0.013±0.011	8786.670±0.972
Zag-7	1.390±0.013	0.014±.014	8761.410±0.970
Zag-10	1.350±0.012	0.009±.013	8756.220±1.287
Zag-11	1.390±0.014	0.013±.015	8708.310±0.969
Zager	1.370±0.015	0.011±0.014	7934.190±1.588
Zag-13	1.370±0.018	0.009±.017	8726.530±0.895
Zag-14	1.410±0.015	0.014±.0017	8981.320±0.986
Khuch	3.340±0.021	0.022±0.020	9696.740±0.899
Purple rice	4.870±0.023	0.019±0.019	9995.340±1.593
Kawa-kreed	2.240±0.017	0.016±0.016	7985.440±0.818
Loual-anzul	1.890±0.016	0.008±0.009	7543.197±1.165
Mushk budji	2.210±0.015	0.012±0.010	6562.560±0.676
Pusa sugandh-3	1.723±0.011	0.015±0.011	6867.680±0.613
Kamad	1.370±0.018	0.007±0.009	6553.570±0.357
Jhelum	1.020±0.017	0.002±0.007	5943.140±0.573
Shalimar rice-1	1.040±0.018	0.002±0.007	5962.653±0.567
Mean	1.76169	0.01063	7367.779
CD	0.033	0.004	0.032
SE(d)	0.016	0.002	0.016

3.8 Conclusion

Food and nutritional security envisages identification of rice varieties with high nutritional value. Rice lines with higher nutrient profiles need to be identified so that people consuming rice diets are supplied with adequate minerals, proteins, carbohydrates and other health promoting agents. It is well known that pigmented varieties are nutritionally better owing to the presence of anthocyanins, which are widely recognized for their antioxidant activity, anticancer, hypoglycemic and anti-inflammatory effects. The present study on the composition and nutritive quality of selected rice genotypes is therefore of great interest. Some of the pigmented varieties investigated in the present study were traditionally being fed to pregnant women in 19th century, for better nutritional value. These genotypes are now proven to be nutritionally better and hence need to be promoted among consumers. The knowledge provided would not only provide the much sought-for information about nutritive value of rice varieties by consumers, but also help to orient the work of investigators involved in varietal selection.

The genotype that recorded maximum protein and fibre content was Mushk budji, while Shalimar rice-1 showed maximum starch and amylose contents. Phenol and anthocyanin concentrations were highest in Purple rice, while carotenoid concentration was highest in Khuch. Shalimar rice 1, Mushk budji, Khuch, Purple rice are distinct in many respects and should be popularized among the farmers for better nutritional security and economic returns. Anthocyanin if extracted from Purple rice (9995.34 µg/g) may be explored for possible use as a natural source of antioxidant agents against ROS in food industry.

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5. References

1. Sanghera GS, Husaini AM, Parray GA, Rather AG, Shikari AB, Wani SA. Generation of cold tolerant CMS lines of rice and identification of maintainers/restorers for hybrid rice development in Kashmir. Indian Journal of Crop Sciences. 2010a; 5:143-146.
2. Husaini AM, Parray GA, Wani SA. Biodiversity of rice germplasm and its conservation: Targets, accomplishments and way forward. In: University Research in Kashmir Rice- A Historical Perspective, SKUAST-Kashmir, 2013.
3. Husaini AM, Bhat ZA, Sanghera GS, Bhat GN, Rather AG. Screening and the development of blast resistant rice varieties for Kashmir valley. Journal of Himalayan Ecology and Sustainable Development. 2011a; 6:1-6
4. Sanghera GS, Husaini AM, Anwar A, Kashyap S. Evaluation of some IRCTN rice genotypes for cold tolerance and leaf blast disease under temperate Kashmir conditions. *Journal of Hill Agriculture.* 2011b; 2:28-32.
5. Ashraf H, Husaini AM, Bhat MA, Parray GA, Khan S, Ganai NA. SSR based genetic diversity of pigmented and aromatic rice (*Oryza sativa* L.) genotypes of the western Himalayan region of India. *Physiology and Molecular Biology of Plants*. 2016; 22:547-555.
6. Anonymous. Hand book of S.R Thimiah New Kalyani publications New Delhi. Edition, D.O.E.S., 2013-14. Economic Survey J&K 2013-14, J&K Govt. Srinagar, 1999, 1.
7. Hodge JE, Hofreiter BT. Method in Carbohydrate chemistry (eds) Whistler, R.L and Bemiller, J.N.

Academic press, New York, 1962.
8. Sadasivam S, Manickam A. In: Biochemical Methods for Agricultural Sciences, Wiley Eastern Ltd., New Delhi, 1992, 184-185.
9. Maynard AJ. Methodsin Food analysis, Academic Press, New York, 1970, 176.
10. Malick CP, Singh MB. Plant Enzymology and Histo-Enzymology. New Delhi, India. Kalyani Publishers, 1980.
11. Jensen A. In: Hand Book of Phytological Method. [Eds. A. Hellebust and J.S. Craigis]. Cambridge University press, London, 1980, 59-70.
12. Swain T, Hillis WE. Physico-chemical and Genetic Analysis of Aromatic Rice (*Oryza sativa* L.) germplasm Agriculturists a Scientific Journal of Krishi Foundation. 1959; 9(1-2):82-88.
13. Juliano BO. A simplified assay of milled rice amylase. Cereal Science Today. 1972; 16(10):334-339.
14. Lone AA, Qazi NA, Ganai1 SA, Bhat TA. Effect of calcium fortification and steaming on chemical, physical and cooking properties of milled rice. African Journal of Agricultural Research. 2013; 8(12):1070-1076.
15. Subudhi H, Meher J, Singh ON, Sharma SG, Das S. Grain and food quality traits in some aromatic long and short grain rice varieties of India. Journal of Food, Agriculture & Environment. 2013; 11(3-4):1434-1436.
16. Thomas R, Wan-Nadiah WA, Bhat R. Physiochemical properties, proximate composition, and cooking qualities of locally grown and imported rice varieties marketed in Penang, Malaysia. International Food Research Journal. 2013; 20(3):1345-1351.
17. Rosniyana A, Jimilah NH, Hajar RI, Shariffah N. Chemical composition and physico-chemical properties of Mahsuri Mutant and Basmati Rice. MARDI Resources Journal. 1995; 23(2):177-181.
18. Resurrection AP, Juliano BO. Tanaka Y. Nitritional content and distribution in milling fractions of rice grain. Journal of Science of Food and Agricultural. 1977; 30:475-481.
19. Yodmanee S, Karrila TT, Pakdeechanuan P. Physical, chemical and antioxidant properties of pigmented rice grown in Southern Thailand, 2011.
20. Deepa G, Singh V, Naidu KA. Nutrient composition and physicochemical properties of Indian medicinal rice. Njavara Journal of Food Chemistry. 2007; 106:165-171.
21. Reddy DK, Bhotmange MG. International Review of Applied Engineering Research, 2013; 4:397-402.
22. Nagi HPS, Sharma S, Sekhon KS. Handbook of Cereal Technology. Kalyani Publishers, India, 2007, 154-155.
23. Husaini AM, Parray GA, Rather AG, Sanghera GS. Performance of elite basmati rice varieties of subtropical India under temperate valley conditions of Kashmir, Genetic resources. International rice research notes, 2009, 0117-4185.
24. Paule CM, Beng KA, Juliano BO, Coffwan WR. Variability in amylase content. *Starke,* 1979; 28:15-22.
25. Gorinstein S, Oscar J, Vargas M, Nicolas O, Jaramillo Salas IA, Ayala ALM *et al*. Journal of European Food Research Technology. 2007; 225:321-328.
26. Tian S, Nakamura K, Kayahara H. Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. Journal of Agric Food Chemistry. 2004; 52:4808-4813.
27. Zho Z, Robertis K, Helli Well S, Blanchanrt C. Distribution of phenolic acids in rice. Journal of Food Chemistry, 2004; 87(3):401-4.
28. Yeum KS, Russell RM. Carotenoid bioavailability and bioconversion. Journal Rev. Nutri. 2002; 22:483-504.
29. Nam SH, Choi SP, Kang MY, Koh HJ, Kozukue N, Friedman M. Antioxidative activities of bran from twenty one pigmented rice cultivars. Food Chemistry. 2006; 94:613-620.
30. Philpott M, Gould KS, Lim C, Ferguson LR. In situ and *in vitro* antioxidant activity of sweetpotato anthocyanins. J Agric Food Chem. 2006; 54:1710-1715.
31. Sutharut J, Sudarat J. Total anthocyanin content and antioxidant activity of germinated colored rice. International Food Research Journal. 2012; 19(1):215-221.
32. Hyun JW, Chung HS. Cyanidin and malvidin from Oryza sativa cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G_2/M phase and induction of apoptosis. J Agric Food Chem. 2004; 52:2213-2217.
33. Tsuda T, Horio F, Uchida K, Aoki H, Osawa T. Dietary cyanidin 3-*O*-*β*-*D*-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia. *J Nutr*. 2003; 133:2125-2130.
34. Tsuda T, Horio F, Osawa T. Cyanidin 3-*O*-*β*-glucoside suppresses nitric oxide production during a zymosan treatment in rats. *J Nutr Sci Vitaminol*. 2002; 48:305-310.
35. Zhang MW, Guo BJ, Zhang RF, Chi JW, Wei ZC, Xu ZH *et al*. Separation, purification and identification of antioxidant compositions in black rice. *Agricultural Science in China,* 2006; 5(6):431-440.
36. Hu C, Zawistowski J, Ling W, Kitts DD. Black rice (*Oryza sativa* L. *indica*) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. J Agric Food Chem. 2003; 51:5271-5277.
37. Abdel-Aal ESM, Yong JC, Rabalski I. Anthocyanin Composition in Blue Black, Purple and Red cereal grain. Journal of Agricultural Food Chemistry. 2006; 54:4696-4704.
38. Abdel-Aal ESM, Hucl P. Composition and stability of anthocyanin in blue grained wheat. Journal of Agricultural Food Chemistry. 2003; 51:2174-2180.

RELATIVE EXPRESSION ANALYSIS OF CSAG GENE DURING DIFFERENT STAGES OF STIGMA DEVELOPMENT IN CROCUS SATIVUS.L (SAFFRON)

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Abstract: Saffron is one of the most expensive spices in the world and its flower is unique in having stigma filled with apocarotenoids. The flower development in saffron is governed by MADS box homeotic genes. The stigma development in saffron flower is promoted by a C-class floral homeotic gene AGAMOUS (CsAG). The expression of CsAG began in yellow stigma and there was a considerable growth in its expression (16 folds) as stigma turned from yellow to Orange stage. The expression of CsAG gene continued to increase from orange stage to scarlet stage, when the stigma had fully developed. It was observed that expression of CsAG gene was in concordance with the development of stigma in saffron flower.

Keywords: AGAMOUS, MADS box, Stigma.

Introduction:

Saffron flower development is governed by specific floral homeotic genes belonging to MADS box family (Wafai et al., 2015). The MADS-box motif is a conserved 56-amino-acid region found within the DNA-binding domain of numerous eukaryotic transcription factors. The MADS-box is a conserved motif found within the DNA binding domains of these proteins and the name refers to four of the originally identified members: -MC M1, AG, QEFA and SRF (Dolan & Fields, 1991). MADS-box genes take their name from the *MINICHROMOSOME MAINTENANCE 1* (*MCM1*) genes in yeast, *AGAMOUS* (*AG*) in *Arabidopsis*, *DEFICIENS* (*DEF*) in *Antirrhinum* and serum response factor (*SRF*) in humans (Riechmann & Meyerowitz, 1997). *AGAMOUS is a C-class gene* involved in stamen and carpel development (Gregis et al., 2008). The AGAMOUS subfamily of MADS-box genes was named after the AGAMOUS gene of A. thaliana and is actively involved in floral development (Yanofsky et al., 1990), which is the only C function gene in this species. In the classical ABC model, the C function defines three different roles: carpel identity, stamen identity and floral meristem determinacy (FMD). The C class TF genes that are involved in the formation of the stamen and carpel also control floral meristem (FM) activity to ensure a definite number of floral organs. In A. thaliana a fourth role for the C function, has been assigned which is the prevention of the faulty expression of A- function genes in the two whorls of reproductive organs (Dreni & Kater, 2013). This is shown by the *A. thaliana* ag mutant, where there is a homeotic conversion of stamens into petals, as a result of the loss of stamen identity and faulty expression of the A function (Dreni & Kater, 2013). In the fourth innermost whorl, in place of a carpel, a new ag flower develops, which in turn develops in its centre another new ag flower. The D class (ovule identity) genes also belong to the AGAMOUS subfamily. FLORAL BINDING PROTEIN 7 (FBP7) and FBP11 of petunia were the first genes to be identified as master regulators of ovule identity. (Colombo et al., 1995). In angiosperms several AGAMOUS subfamily genes have been reported and also characterized, such as the monocots rice (Oryza sativa) and maize (Zea mays) which imply that these genes have functional conservation within flowering plants and they function as master regulators of stamen, carpel and ovule identity (Pinyopich et al., 2003). The number of AGAMOUS subfamily genes may differ between different species and typically show various degrees of redundancy. In saffron CsAG plays an important role in development of stigma and three AP1-, Five PI-, two AP3-, two AG- and two SEP-like MADS Box genes from *Crocus sativus* L were isolated (Tsaftaris et al., 2005). In this study, we have studied the relative expression of AGAMOUS gene during different stages of stigma development.

Methodology:

Sample Collection and storage

The samples were collected at three different stages of saffron flower on the basis of stigma development. The three stages were yellow stigma, orange stigma and scarlet stigma (Fig 1). The samples were collected from the field and immediately dipped in liquid nitrogen. For long term storage these samples were stored at -80^{o}C.

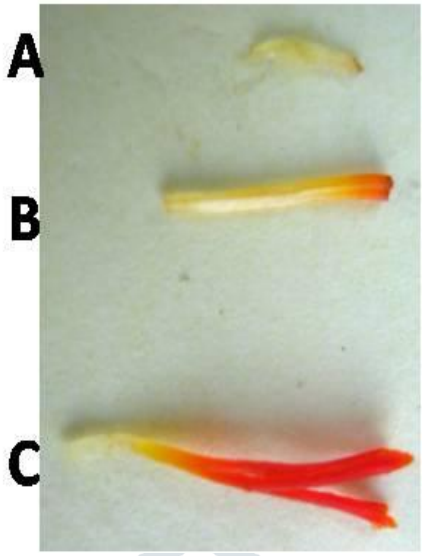


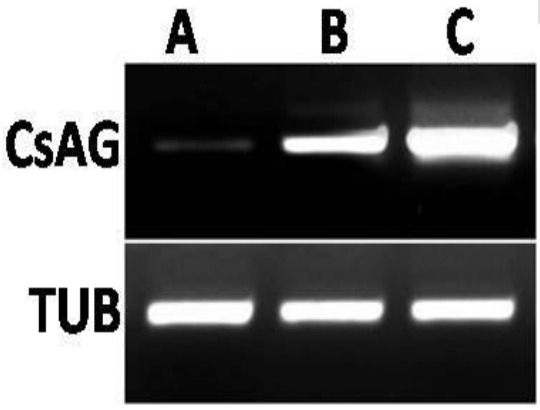
Fig 1: Three different stages of stigma development – A)yellow, B) Orange, C) Scarlet

RNA Extraction and Quantitative Real time PCR

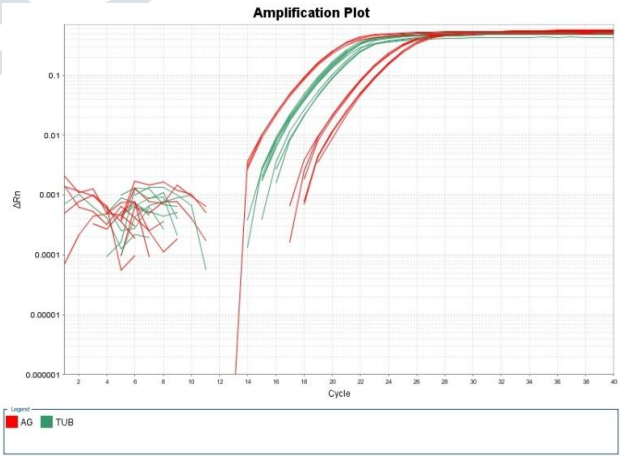
RNA was extracted from 100mg stigma in all three stages using Trizol reagent. 1µg/mL total RNA was used for synthesis of first strand cDNA using first strand cDNA synthesis kit (Bangalore Genei). The semi quantitative expression analysis was performed by Reverse transcription PCR using the primers CsAG-F **5´-GGCGGATCCATAGCAATAAGGTACCCAGTCAC-3´** and CsAG-R **5´-CGCCTCGAGAAGCTTCCTTCAAGCGAACTTG-3'** for CsAG gene and (5'-TGATTTCCAACTCGACCAGTGTC-3') and (5'-ATACTCATCACCCTCGTCACCATC-3') for tubulin gene. The amplification was carried out at 94C for 2 min, 25 cycles of 94C for 45sec, 54C for 45sec, 72C for 1 min, final extension of 72C for 5 mins. The PCR products were run on 2% agarose gel and the bands were visualized using EtBr stain. The real time PCR was done by same primers as in reverse transcription PCR. Tubulin gene expression was used as an internal control for normalization. The amplification was carried using SybrGreen at 94C for 2 min, 40 cycles of 94C for 15sec, 54C for 15sec, 72C for 20 sec, final extension of 72C for 2 mins. Advanced Relative quantification between different stages was done through $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Result and discussion:

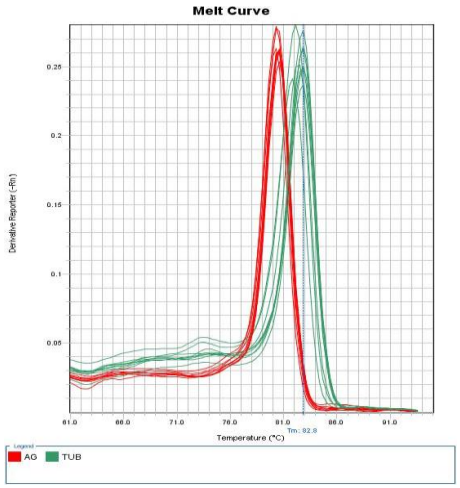
Expression analysis of CsAG gene during 3 stages of stigma development i.e., yellow, orange, and scarlet was done through reverse transcription PCR and real time PCR to find out the relative change in expression during three stages of stigma development. The reverse transcription semi quantitative PCR revealed that the expression of CsAG gene was little at yellow stage and there was a gradual increase in its expression in orange and scarlet stage (Fig 2A). Real time PCR showed that there was an increase in expression (16 folds) of CsAG gene from yellow to orange stage of stigma development. The expression level of CsAG gene further increased (6.25 folds) from orange to scarlet stage of stigma development. Hence the expression level during three stages of stigma development (yellow:orange:scalet) was observed to be 1:16:100. (Fig. 2D)



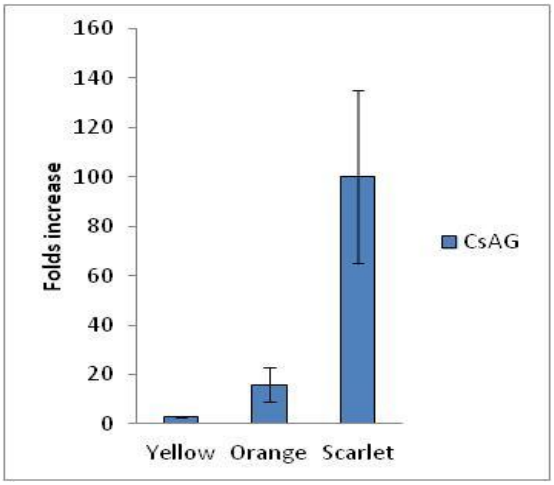
A)



B)



C)



D)

Fig 2: (A) Reverse Transcription PCR products of CsAG and TUB; (B)Amplification curve of CsAG and tubulin gene (C) Melting curve for CsAG and TUB, (D) Relative expression of CsAG gene during different stages of stigma development.

AGAMOUS gene is involved in development of stamens and stigma in saffron flower (Tsaftaris et al., 2005). Its expression varied at different stages and different organs of the flower during the course of its development. AG expression has been reported to be very high in developing stamens and stigma (Meyerowitz et al., 1991). In the present study, least expression of CsAG in yellow stage can be explained by the fact that the expression of AP1 gene is high at this stage, which negatively regulate the CsAG expression (Lamb et al., 2002). The abrupt increase in CsAG expression from yellow to orange stage of stigma development can be explained on the fact that, at the orange stage, the stigma development takes a high pace and AP3-PI heterodimers positively regulate the CsAG expression (Wafai et al., 2015). At Later stage, when flower is open and stigma are scarlet in color, the expression of AP1 gene is minimal due to inactivation by AP3-PI heterodimer which, in turn, results in highest expression of CsAG gene (Jens. 2006).

This is a preliminary work on the expression of floral homeotic genes in saffron. More work need to be done on the expression pattern of other homeotic genes like AP1, LFY, PI, AP3, CAL and SEP, which will give a holistic picture of the regulation of these floral genes and their effect on morphology and development of the saffron flower organs. This study can pave a way for *in vitro* flowering of saffron by activation of particular key genes. We have already studied the expression of CsAP3 gene and CsNAP gene earlier and with complete analysis of expression pattern of all floral homeotic genes, we may understand the flower development pathway in saffron in detail.

References:

[1] Colombo L, Franken J, Koetje E, van Went J, Dons H J, Angenent G C and van Tunen A J. The petunia MADS box gene FBP11 determines ovule identity. 1995. *Plant Cell* **7**: 1859–1868.

[2] Dolan J W and Fields S. Cell-type-specific transcription in yeast, Biochim. Biophys. 1991. Acta 1088:155-169.

[3] Dreni L and Kater M M. MADS reloaded: evolution of the AGAMOUS subfamily genes . 2013. New Phytol, 201(3):717–732.

[4] Gregis V, Sessa A, Colombo L and Kater M M. AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in Arabidopsis. 2008. Plant J. 56:891–902.

[5] Jens F S. Direct regulation of the floral homeotic APETALA1 gene by APETALA3 and PISTILLATA in Arabidopsis. 2006. The Plant Journal 46:593–600.

[6] Lamb R S, Hill T A, Tan Q K G and Irish V F. Regulation of APETALA3 floral homeotic gene expression by meristem identity genes. 2002. Development 129:2079-2086.

[7] Livak K J and Schmittgen T D. Analysis of relative gene expression data using real- time quantitative PCR and the 2-ΔΔcp Method. 2001. Methods 25:402–408.

[8] Meyerowitz E M, Bowman J L, Brockman L L, Drews G N, jack T, Sieburth L E and Weigel D. A genetic and molecular model for flower development in *Arabidopsis thaliana. 1991.* Development Supplement 1:157-167.

[9] Pinyopich A, Ditta G S, Savidge B, Liljegren S J, Baumann E, Wisman E and Yanofsky M F. Assessing the redundancy of MADS-box genes during carpel and ovule development. 2003. *Nature* **424**: 85–88.

[10] Riechmann J L and Meyerowitz E M. MADS domain proteins in plant development. 1997. *Biol. Chem.* **378**:1079–1101.

[11] Tsaftaris A S, Pasentsis K, and Polidoros A N. Isolation of a differentially spliced C-type flower specific AG-like MADS-box gene from Crocus (Crocus sativus) and characterization of its expression. 2005. Biol. Plant. 49:499– 504.

[12] Wafai A H, Bukhari S, Mokhdomi T A, Amin A, Wani Z, Hussaini A, Mir J I & Qadri R A. Comparative expression analysis of senescence gene CsNAP and B-class floral development gene CsAP3 during different stages of flower development in Saffron (Crocus sativus L). 2015. Phy & Mol Bio of Plants. 21(3):459-463.

[13] Yanofsky M F, Ma H, Bowman J L, Drews G N, Feldmann K A and Meyerowitz E M. The protein encoded by the*Arabidopsis* homeotic gene *agamous* resembles transcription factors. 1990. *Nature* **346**:35–39.

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Screening of important bean genotypes/collections for resistance against Common Bean Mosaic Virus using molecular markers

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Abstract
Bean Common Mosaic Virus (BCMV) causes one of the most serious diseases affecting common bean production. This potyvirus is spread non-persistently by aphids and is also seed-borne. In present investigation out of 132 genotypes selected for screening for BCMV only 8 genotypes (WB-399, WB-640, WB-359 & WB-375, WB-494, WB-933 & WB-939, WB-335) were found phenotypically resistant against it, which was later ascertained by molecular analysis by different microsatellite markers. The variable number of bands produced by different primers ranged from 5 to 22 with an average of 11.4 bands per primer and with level of polymorphism 24.5%. Among the molecular markers used, SSR marker named 835 produced a maximum of 6 alleles, followed by marker 841 then by marker 827 then by BMD-1 followed by ROC-11then followed by marker EIF-4E then followed by SBD-5 and finally by SW-13 that produced 3 alleles each. The polymorphism information content (PIC) was calculated for all the markers, and was highest for Primer BMD-6 which exhibited PIC value of 0.49 and the lowest for the primer BMD-2 (0.22). Hence, primer BMD-6 was observed to be highly informative and serve as an effective and useful marker to determine the genetic differences among the common bean accessions and also to study the phylogenetic relationships.

Keywords: bean, genotypes, resistance, virus, markers

Introduction
In *Phaseolus vulgaris* two main types of symptom occur, depending on virus strain and host genotype: the common mosaic often associated with leaf malformation, and 'black root' characterized by the systemic necrosis and plant death [1]. This virus causes mosaic in most other susceptible *Phaseolus* species [2] [3]. A virus with flexuous filamentous particles *c.* 750 nm long and 12-15 nm wide, containing single-stranded RNA, transmitted by mechanical inoculation, by several aphid species in a non-persistent manner, and through seed and pollen. The virus induces the formation of cylindrical inclusions in the cytoplasm of infected cells. In nature it is mainly restricted to *Phaseolus* species, especially *P. vulgaris,* being found wherever this legume is grown. *Phaseolus vulgaris.* A set of differential varieties possessing the dominant necrosis gene and/or different strain-specific recessive genes is available for strain identification [1]. The virus is transmitted in a non-persistent manner by several aphid species, notably *Acyrthosiphon pisum, Aphis fabae* and *Myzus persicae* [4], [5]. Other reported vector species include *Aphis gossypii, A. medicaginis, Hyalopterus atriplicis, Macrosiphum ambrosiae, M. pisi* and *M. solanifolii* [6]. Most of these aphid species do not colonize *P. vulgaris* but transmit the virus efficiently as winged migrants. Aphids acquire the virus optimally in probes of 15-60 sec and transmit it within 1 min [7],[5],[8].The high incidence of seed transmission is probably the most important factor affecting initial crop infection and the world-wide distribution of the virus. Depending upon the bean genotype and virus strain tested, up to 83% of the seed produced by infected plants may give rise to common mosaic-affected plants [9], [10], [11]. The virus is not appreciably affected during prolonged storage of the seed up to 30 years [12]. The virus is located mostly in the embryo [13]; virus in the seed coat is inactivated during seed maturation [14]. [15]. Classifies the virus in Subdivision I of the potyvirus group, according to the morphology of the cylindrical (pinwheel) inclusions induced in the cytoplasm of infected cells.

Materials and methods
Plant material
The material for the present study comprised of 132 germplasm accessions of common bean (both local and exotic). The pedigree of the lines used is given in Annexure-I. The material

represented diverse market classes of common bean based on growth habit, seed shape, colour and pod characters. The present investigation was undertaken during 2014-2015 at Centre for Plant Biotechnology, SKUAST-K, Shalimar.

Screening of genotypes for identification of resistant genotypes for BCMV

The screening of genotypes was done by artificial inoculation of genotypes as described by [16].

Estimation of genetic diversity in bean lines using microsatellite (SSR) markers

Genomic DNA extraction

Plant DNA was isolated using CTAB (Cetyl Trim ethyl Ammonium Bromide) method as modified by [17].

Selection of primers

The primer markers utilized in the present study were selected on the basis of literature available. Among these, a set of 13 microsatellite markers were selected.

PCR amplification

In vitro amplification using polymerase chain reaction (PCR) were performed in a 96 well microtiter plate in a Bio-Rad or Eppendorf master cycler using 75 ng of genomic DNA of each genotype in a final volume of 20 µl per reaction. The stock and final concentration of different components that were used in PCR is given in table 1& 2.

Visualization of PCR products and Scoring of primer allele profile

The primer allele sizes were determined by the position of bands relative to the DNA ladder. Total number of alleles were recorded for each microsatellite marker in all the genotypes under study by giving the number to amplified alleles as 1, 2, 3, 4 and so on. Number 1 was given to the allele having highest molecular weight. The amplified bands were recorded as 1 (band present) and 0 (band absent) in a binary matrix. The accessions that did not show any amplification were scored as missing values if amplification were not repeated and as null alleles if the amplification was repeated 2-3 times. If the band appeared in the negative control the whole PCR reaction experiment were discarded and repeated again.

Table 1: Stock and final concentration of different components used in PCR

Components	Stock Conc	Volume (µl)	Final Conc
Water	-	3.8	-
PCR buffer	10X*	2.0	1X
$MgCl_2$	25mM	1.2	1.5mM
dNTPs	1mM	4.0	100µM
Primer Forward	5µM	1.0	0.25µM
Primer Reverse	5µM	1.0	0.25µM
Taq Polymerase	5U/µl	0.2	1Unit
DNA template	15ng/µl	5	75ng
Total		20	

*10X PCR buffer: 10mM TrisHCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01 % Gelatin.

The reaction mixture was overlaid with a drop of low molecular weight mineral oil (SIGMA) and placed in 96 well thermal cycler. Amplification were performed using temperature profile mentioned in Table 1.

Table 2: Temperature profile used in PCR

Step	Temperature	Time (minutes)	No. of cycles
Initial denaturation	94	4	1
Denaturation	94	1	35
Annealing	50-60	2	
Elongation	72	2	
Final Extension	72	7	1
Hold	12		

Polymorphism information content (PIC) values and effective multiplex ratio (EMR) and marker index (MI)

The PIC values as described by [18] were used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. PIC values for each of the primer were estimated using formula given by [19].

$$PIC = 1- \sum_{i=1}^{n} (P_{ij})^2$$

Where P_{ij} is the frequency of j^{th} allele in i^{th} primer and summation extends over 'n' patterns. PIC is synonymous with the term 'gene diversity' as described by [20]. The PIC takes into account not only the number of alleles that are expressed but also the relative frequencies of those alleles [21].

Number of loci (L): number of loci in case of RAPD is equal to the total number of bands ($n_p + nn_p$) obtained. Number of loci per assay unit: nu = L/U. Fraction of polymorphic loci $\beta = n_p/n_p + nn_p$. Effective multiplex ratio E = nuβ. Marker index MI = PIC × β × α. [22].

Experimental findings

The present investigation entitled "Identification and morpho-molecular characterization of resistant bean common mosaic virus (BCMV) genotypes of common bean (*Phaseolus vulgaris* L.)" was undertaken to generate the information on No. of genotypes found resistant against BCMV from 132 genotypes using two susceptible checks *viz.*, SR-1 and WB-967. Genetic divergence was observed on the basis of morphological data in 10 common beans genotypes. Besides it, molecular characterization of resistant bean common mosaic virus genotypes of common bean (*Phaseolus vulgaris* L.) was undertaken to elicit information on genetic polymorphism, similarity matrix, polymorphic information content, and cluster analysis.

Screening

Out of 132 genotypes, using two known susceptible checks *viz.*, SR-1 and WB-967, only 8 genotypes (WB-399, WB-640, WB-359 & WB-375, WB-494, WB-933 & WB-939, WB-335) were found resistant against BCMV, which were then objected to calculate molecular data (Fig 1 & 2; Table 3& 4).

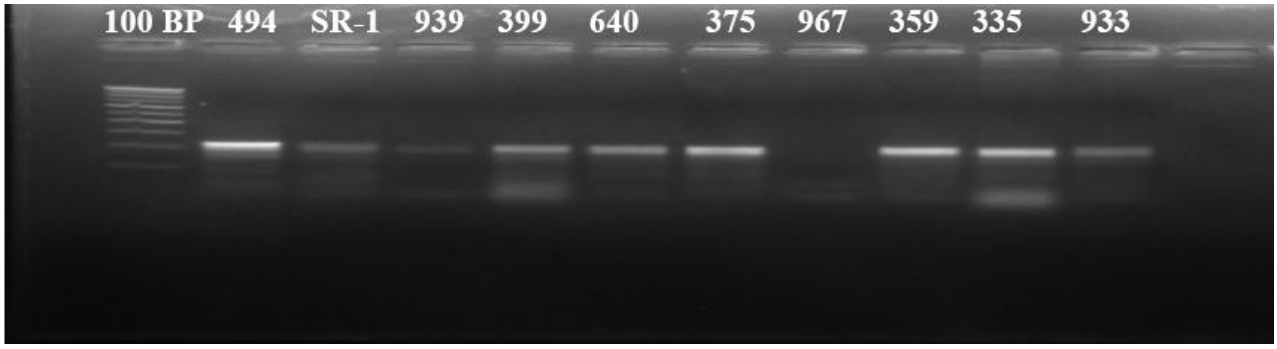

Fig 1: Ethidium bromide stained gel picture showing genetic relationship among 10 common bean (*Phaseolus vulgaris* L.) genotypes using primer BMD-3

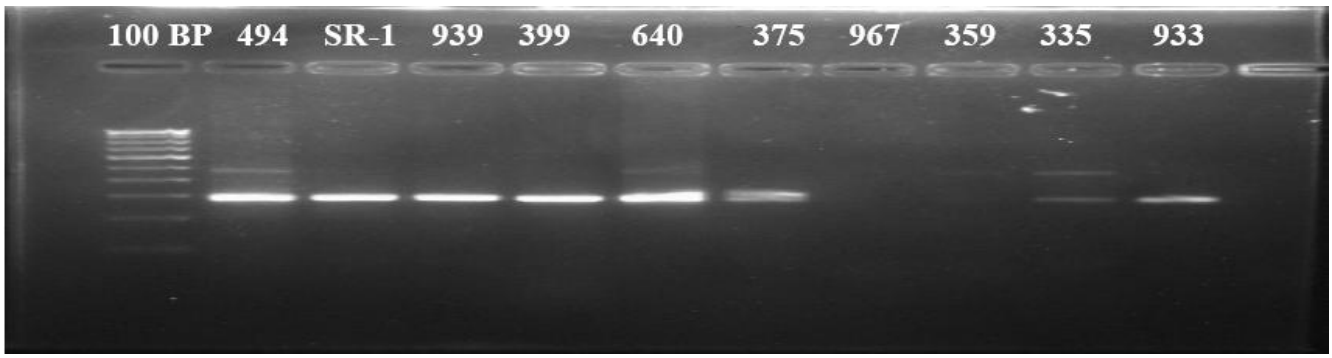

Fig 2: Ethidium bromide stained gel picture showing genetic relationship among 10 common bean (*Phaseolus vulgaris* L.) genotypes using primer ROC-11

Table 3: Percentage of polymorphism obtained by PCR amplification of DNA in 10 common beans (*Phaseolus vulgaris* L.) genotypes

Primer	Total No. of bands	No. of polymorphic bands	Percentage of polymorphism
841	13	3	23
835	17	8	47
827	5	2	40
BMD-3	9	0	-
BMD-2	8	0	-
BMD-1	12	3	25
BMD-6	6	0	-
BMD-4	16	6	37.5
ROC-11	13	4	30.7
Eif-4e	9	0	-
SG6	8	0	-
SBD-5	22	13	59
SW-13	10	1	10
Total	148	40	
Mean per primer	11.4	3.07	

Table 4: Polymorphic information content (PIC) of 13 primers across 10 genotypes of common bean (*Phaseolus vulgaris* L.)

Primer	Polymorphic information content (PIC)	Effective multiplex ratio (EMR)	Marker index (MI)
841	0.44	0.09	0.039
835	0.32	0.08	0.025
827	0.29	0.06	0.017
BMD-3	0.34	0	0
BMD-2	0.22	0	0
BMD-1	0.28	0.12	0.033
BMD-6	0.49	0	0
BMD-4	0.24	0.30	0.072
ROC-11	0.30	0.13	0.039
EIF-4E	0.38	0	0
SG6	0.38	0	0
SBD-5	0.26	0.43	0.111
SW-13	0.44	0.03	0.013
MEAN	0.39	0.09	0.035
CD (0.05%)	0.04	-	-

Discussion

The morphological analysis revealed that BCMV is present in Kashmir valley with high incidence (Fig 1&2, Table 3& 4). Out of 132 genotypes only 8 were found to be resistant against BCMV [16]. Similarly, based on various morphological characteristics, various brinjal genotypes/collections were observed to exhibit variable responses to pest (*Leucinodes orbonalis* Guenee) [23]. In the present study, microsatellite markers were used to determine the extent of diversity among 10 genotypes of common bean. This findings can have implications for detailed studies on genetic diversity among genotypes [24]. Further, the resistance breeding based on molecular approaches intergraded with conventional one help in green revolution in food security [25], [26]. The Molecular markers such as SSR can be used to study genetic diversity among genotypes [27], [28], [29]. Thirteen primers amplified in 10 genotypes produced 37 alleles which were used to generate marker profiles. The number of bands produced by different primers ranged varied from 5 to 22 with an average of 11.4 bands per primer and level of polymorphism 24.5%. Among the primers used, 835 produced a maximum of 6 alleles, followed by 841, 827, BMD-1, ROC-11, EIF-4E, SBD-5 and SW-13 produced 3 alleles each. [30]. Found that from 20 SSR markers evaluated using 85 accessions, the number of alleles per locus ranged from 3 to 10 with a mean of 7. They also recorded a lower observed heterozygosity (He) of 0.026 compared to the expected heterozygosity (He) of 0.622, suggesting that it is also an inbreeding crop. Further, the findings of higher polymorphism detected by SSRs are in agreement with earlier findings of [31], [32], [33] and [34]. In contrast, [35] reported an average of 11 alleles per locus in an SSR analysis of a worldwide common bean collection, whereas [36] reported over 72 alleles in an SSR analysis, with an average of 18 alleles per locus, in an international collection of common beans from Andean and Mesoamerican gene pools. SSRs are characterized by their hyper variability, abundance, reproducibility, Mendelian mode of inheritance and co-dominant nature [37]. Amplification success and polymorphism declines with increased genetic distance. The high level of polymorphism of microsatellite markers and their wide cross-species transferability make these new markers useful for mapping and molecular characterization of *Phaseolus* species [37].

Polymorphism information content (PIC) value of each microsatellite marker is a measure of marker diversity. PIC value provides an estimate of discriminatory power of a locus by taking into account not only the number of alleles expressed, but also the relative frequency of those alleles. The polymorphism information content (PIC) was calculated for all the markers. PIC was highest for Primer BMD-6 exhibited PIC value of 0.49 and the lowest for the primer BMD-2 (0.22). Hence, primer BMD-6 was observed to be highly informative in the present study. This indicated that the Primer BMD-6 might serve as an effective and useful marker to determine the genetic differences among the common bean accessions and to study the phylogenetic relationships. In previous report for 35 North American Soybean Ancestors (NASA), five AFLP primer-pairs produced 90 polymorphic (27%) and 242 monomorphic AFLP fragments. The polymorphic information content (PIC) scores ranged from 0 to 0.49. The polymorphism observed in SSR markers among the common bean genotypes in the present study demonstrated the effectiveness of this method in determining genetic variation. All the SSR markers used were found to be highly informative in revealing the genetic diversity analysis. The result of the present study are in accordance with the earlier reports of [38, 34, 39, 40].

Summary and conclusion

The present study revealed that BCMV is present in Kashmir valley of India in high persistence as morphological study was affirmed by molecular analysis.

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References

1. Drijfhout, *Genetic Interaction between* Phaseolus vulgaris *and Bean Common Mosaic Virus, Doctoral Thesis*, Centre Agric. Publ. Docum. Also in *Agric. Res. Rep., Wageningen*. 1978; 98:872.
2. Ainsworth, The identification of certain viruses found infecting leguminous plants in Great Britain. *Annuals of Applied Biology*. 1940; 27:218.
3. Kaiser & Mossahebi, Natural infection of mung-bean by Bean Common Mosaic Virus. *Phytopathology*. 1974; 64: 1209.
4. Kennedy Day, Eastop A. *Conspectus of Aphids as Vectors of Plant Viruses*, London, Commonwealth Institute of Entomology. 1962, 114.
5. Zettler FW. Aphid population in central New York as related to epiphytology of bean common mosaic virus *Cornell University Ph.D. Thesis*, 1966.
6. Zaumeyer WJ, Thomas HRA. Monographic study of bean diseases and methods for their controle. *Technical Bultien. U.S. Department of Agriculture.* 1957; 868:255 pp.
7. Van der Want, Onderzoekingen over Virusziekten Van der boon *Phaseolus vulgaris* L. *Doctoral thesis Wageningen Agricultural University the Netherlands.* 1954, 85-84.
8. Zettler FW. The heterogeneity of Bean leaves as source of bean common mosaic virus for aphids. *Phytopathology.* 1969; 59:1109.
9. Reddick & Stewart, Transmission of virus of bean mosaic in seed and observations of thermal death point of seed and virus. *Phytopathology*. 1919; 9:445.
10. Schippers, Transmission of bean common mosaic virus by seed of phaseolus vulgaris l. Cultivar beka *Plant biology*. 1963; 12:433.
11. Morales & Castaño. Seed transmission characteristics of selected bean common mosaic virus strains in different bean cultivars. *Plant Diseases.* 1987; 71:51.
12. Pierce WH, Hungerford CW. A note on longevity of bean Mosaic Virus. *Phytopathology*. 1929; 19:605.
13. Provvidenti R, Cobb ED. Seed transmission of Bean Common Mosaic Virus. *Plant Dieases.* 1975; 59:966.
14. Hagita T, Tamada T. Detection of Bean Common Mosaic Virus in French bean seeds by immune electron microscopy. *Bulletin Hokkaido Express. 1984;* 51:83.
15. Edwardson JR. Some properties of the potato virus Y group. Florida Agricultural Experimental Station Monographs Series 4. 1974, 398.
16. Wani AB, Bhat MA, Mir ZA, Dar NA, Sofi PA. Screening of Genotypes for Identification of Resistant Genotypes for BCMV. Research Journal of Agricultural Sciences. 2017; 8(2):320-323
17. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Anand RW. Ribosomal DNA spacer length polymorphism in

barley: Species diversity, chromosomal location and population dynamics. *Proceeding of National Academy of Science USA.* 1984; 91:5466-5470.
18. Botstein D, White RL, Skolnick MH, Davis RW. Construction of a genetic map in man using restriction fragment length polymorphism. American Journal of Human Genetics. 1980; 3:314-331.
19. Nei M. Analysis of genetic diversity in subdivided population. *Proceedings of the National Academy of Sciences of the United States of America.* 1973; 70:3321-3323.
20. Weir BS. Genetic data analysis: Methods for discrete population genetic data (Sinauer Associates, Inc, Sunderland, MA), 1990.
21. Smith JSC, Chin ECL, Shu H, Smith OS, Smith OS, Wall SJ, *et al*. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. *Theoretical and Applied Genetics.* 1997; 95:163-173.
22. Powell W, Morganate M, Andre C, Hanafey M, Rafalski A. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding.* 1996; 2:225-238.
23. Dar SA, Wani AR, Mir SH, Nehru RK, Jeelani MI. Relationship between morphological characters of different brinjal genotypes and extent of infestation by *L. orbonalis*, Guenee. *Green farming*. 2014; 5(6):1096 1100
24. Masi P, Logozzo G, Donini P, Zeuli PS. Analysis of genetic structure in widely distributed common bean landraces with different plant growth habits using SSR and AFLP markers. *Crop Science.* 2009; 49:187-199.
25. Dar SA, Wani AB, Kandoo AA. Genetic engineering and insect resistance. Journal of Entomology and Zoology Studies. 2017; 5(3):1175-1184.
26. Dar SA, Wani AB. INSECT. International Research Publication House. Head Office: B-2/84, Ground Floor, Rohini Sector-16, Delhi-110089, INDIA. www.irphouse.com. Book. Genetic Engineering. Chapter. 2016; 22:309-339.
27. Kumar J, Vijeshwar V, Shahi AK, Qazi GN. Development of simple sequence repeat markers in Cymbopogon species. *Planta Medica*. 2006; 73:262-266.
28. Menz MA, Klein RR, Unruh NC, Rooney WL, Klein PE, Mullet JE. Genetic diversity of public inbreds of *Sorghum* determined by mapped AFLP and SSR markers. *Crop Science.* 2004; 44:1236-1244.
29. Tams SH, Melchinger AE, Bauer E. Genetic similarity among European winter triticale elite germplasms assessed with AFLP and comparisons with SSR and pedigree data. *Plant Breeding.* 2005; 124:154-160.
30. Buso GSC, Amaral ZPS, Brondani RPV, Ferreira ME. Primer note: microsatellite markers for the common bean. *Molecular Ecology Notes.* 2006; 6:252-254
31. Badjakov I, Todorovska E, Kondakova V, Boicheva R, Atanassov A. Assessment the genetic diversity of Bulgarian raspberry germplasm collection by microsatellite markers. Journal of Fruit and Ornamental Plant Research. 2006; 14:61-76.
32. Blair MW, Diaz JM, Hidalgo R, Diaz LM, Duque MC. Microsatellite characterization of Andean races of common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics.* 2007; 119(6):955-972.
33. Rossi M, Bitocchi E, Bellucci E, Papa R. Linkage disequilibrium and population structure in wild and domesticated populations of *Phaseolus vulgaris* L. *Evolutionary Applied*. 2009; 2:504-522.
34. Burle ML, Fonseca JR, Kami JA, Gepts P. Microsatellite diversity and genetic structure among common bean (*Phaseolus vulgaris* L.) landraces in Brazil, a secondary center of diversity. *Theoretical and Applied Genetics.* 2010; 121:801-813.
35. Blair MW, Giraldo MC, Buendia HF, Tovar E, Duque MC, Beebe SE. Microsatellite marker diversity in common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics.* 2006; 113:100-109.
36. Blair MW, Diaz LM, Buendia HF, Duque MC. Genetic diversity, seed size association's andpopulation structure of a core collection of common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics.* 2009; 119(6):955-972.
37. Sicard D, Nanni L, Porfiri O, Bulfonn D, Papa R. Genetic diversity of *Phaseolus vulgaris* L. and *P. coccineus* L. landraces in central Italy. *Plant Breeding.* 2005; 124(5):464-472.
38. Blair MW, Pedraza F, Buendia HF, Gaitan-Solis E. Development of a genome-wide anchored microsatellite map of common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics.* 2003; 107:1362-1374.
39. Yu K, Park SJ, Poysa V, Gepts P Integration of simple sequence repeats (SSR) markers into a molecular linkage map of common bean (*Phaseolus vulgaris* L.). Journal of Heredity. 2000; 91:429-434.
40. Gaitan SE, Duque MC, Edwards KJ, Tohme J. Microsatellite repeats in common bean (*Phaseolus vulgaris* L.): Isolation, characterization, and cross-species amplification in *Phaseolus* ssp. *Crop Science.* 2002; 42:2128-2136.

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In vitro PROPAGATION OF CHINAR (*Platanus orientalis* L.) USING NODE AND INTERNODE EXPLANTS

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ABSTRACT

The aim of present study was to standardize a micropropagation protocol for Chinar (*Platanus orientalis* L.) using nodes and internode segments as explant materials. The sterilization treatment with 0.1% mercuric chloride for 10 min showed highest survival percentage in nodal explants (40.2) and internodal explants (51.9). Internodal explants survived sterilant treatment better than nodal explants. For shoot regeneration, MS (Murashige and Skoog medium) medium supplemented with indole butyric acid (IBA) (0.25 mg L^{-1}), benzyl adenine (BA) (4 mg L^{-1}) proved best for both nodal and internodal explants. The maximum number of shoots (9.7 explant^{-1}) greater shoot length (3.9 cm) was obtained from the internodal explants on MS medium supplemented with BA (4 mg L^{-1}) + IBA (0.25 mg L^{-1}). MS medium supplemented with IBA (1 mg L^{-1}) and BA (2 mg L^{-1}) showed 100% rooting percentage as well as highest number and length of roots (15.7 shoot^{-1}, 3.7 cm, respectively). The internodal explants showed better response than nodal explant. Rooted plantlets were then transferred to perforated plastic pots containing sterilized mixture of sand and soil (1:1) ratio, and grown in greenhouse. The survival percentage after 4 weeks in greenhouse was recorded as 62.3%.

Key words: Chinar, *Platanus orientalis*, tissue culture, micropropagation, *in vitro* propagation

INTRODUCTION

Chinar (*Platanus orientalis* L.) is a tree species belonging to the family Platanaceae. It is the only species of Platanaceae found in India and its growth is confined to Jammu & Kashmir state (Kozgar and Khan, 2011). It can attain a height of about 30 m and a girth upto 12 m. The bark is greyish and leaves are borne alternately on stem, deeply 5-7 lobed 12-20 cm in length, and palmate or maple-like with long stalk. Petiole is quite long and length ranges between 3 and 8 cm. Flowers are dense spherical heads, monoecious and unisexual. Fruits are sphere-shaped, compound, and 2 to 6 of them are found on a long leafstalk. Presumably chinar originated in geological times from divergence involving the intercontinental disjunction and other barriers (Sert *et al.*, 2008; Pilotti *et al.*, 2009). It is found naturally in the forest lands of Turkey and is widely used in landscape design (urban open green spaces, parks, arboreta, water fronts, industrial areas, shade bearer and street tree), and there are numerous examples of these being protected as natural monumental trees in Turkey (Zencirkiran, 2010; Zencirkiran and Erken, 2012). It spread wide across a region of cool climate with sufficient water. The chinar trees have spread to Asia, especially the Western Himalayan

region of India, perhaps from Greece. Chinar has become an integral part of Kashmiri culture and a heritage tree that has survived for ages. Kashmir houses Asia's largest and oldest chinar tree which is 700-years-old, located at Chattergam (Budgam) with a circumference of 31.85 m and height of 14.78 m (Wadoo, 2007).

Chinar, a multipurpose tree, is of immense aesthetic importance owing to its beauty and magnificence. Its leaves and bark are used as medicine, the wood is used for making delicate furniture items and the twigs and roots are used for making dyes (Sharma, 2008). Jammu and Kashmir is ecologically fragile and ranks 20th on environment sustainability index (ESI) with ESI score of 40.0 which indicates greater pressure on its ecosystem, higher pollution and vulnerability to environmental predicaments (*http://www.greenindiastandards.com/jammu-kashmir.php?stateid =14*). Chinar population in India is declining due to illegal felling and needs immediate efforts for its conservation (*https://www.kashmirmonitor.in/Details/123741/chinar-the-dying-heritage*). The number of chinar trees in J&K state has reduced to over half - from 42,000 in 1970 to less than 27,000 (Kozgar and Khan, 2011).

Chinar is propagated by generative and vegetative methods. Propagation through seeds (after stratification) is the first option, however, the rate of germination is poor (30-40%) [Anonymous, 2003; Hartman *et al.,* 2011]. Propagation by stem cutting is the second option; however, rooting potential is affected by the factors like genotype, type of cutting (basal median), position of cutting-donor shoots on mother plant, cutting stem diameter, humidity and temperature of rooting medium, time of cutting collection, etc. (Grolli *et al.,* 2005; Dirr and Heuser, 2006; Khosrojerdi *et al.,* 2006). These limitations can be overcome through development of *in vitro* propagation protocol. However, the availability of information about the micropropagation of *Platanus* species is very scanty (Guoqiang *et al*., 2003). Most of the studies pertain to *P. acerifolia* (London plane tree), a hybrid of *Platanus orientalis* (oriental plane) and *P. occidentalis* (American sycamore) [Gjuleva and Atanasov, 1994; Liu *et al*., 2002; Liu and Bao, 2003]. Perusal of literature reveals that there is only one report on micropropagation of *P. orientalis* through use of leaf explant and no protocol is available while using node or internodes as explant. This is not only a limitation for propagation of this plant, but also a major bottleneck in its genetic improvement using plant tissue culture methods. The present study was, therefore, aimed at to standardize an *in vitro* micropropagation protocol for chinar (*P. orientalis*). This is not only a limitation for propagation of this plant, but also a major bottleneck in its genetic improvement using plant tissue culture methods.

MATERIALS AND METHODS

Explant source

The present study was conducted in the Centre for Plant Biotechnology, SKUAST-Kashmir, Shalimar (J&K). *Platanus orientalis* trees (of more than 300 years age) growing at the campus were chosen as mother trees for explant collection. The first and second nodal and internodal segments were collected from the tip of disease free juvenile branches of plus trees. The succulent and actively growing nodes were chosen for the purpose while nodes older than 40 days were hard. Explants were washed with running tap water 2-3 times followed by 2-3 rinses with double distilled water. These were then surface sterilized by mercuric chloride (0.1-0.3%) for different time durations (1-10 min) followed by washing of explants with autoclaved water. Each treatment constituted of 60 explants and was repeated twice. Mortality percentage of explants, contamination percentage, and survival percentage were recorded for each treatment.

Culture conditions

All chemicals and reagents were procured from HiMedia (India) while borosilicate glassware was procured from Borosil Glassworks Ltd. (India). The sterilized explants were cultured on ium

(Murashige and Skoog, 1962) supplemented with B_5 vitamins, 3% sucrose and 0.8% agar agar. Different concentrations and combinations of auxins and cytokinins were used for plant regeneration (Table 2). For every combination, 10 test tubes with 1 explant each were inoculated and the experiment was repeated twice. The pH of medium was adjusted to 5.8 prior to adding 0.8% agar. The cultures were incubated at 25±2˚C in sterilized room with a 16/8h light/dark regime, 75% relative humidity and a light intensity of 3500 lux. The *in vitro* developed plantlets were removed from the agar medium and washed gently to record the observations. For shoot regeneration, the parameters recorded were shoot regeneration percentage, shoot length (cm) and number of shoots regenerated explant^{-1} (Husaini and Abdin, 2007). For rhizogenesis, the parameters studied were root regeneration percentage, root length (cm) and number of roots regenerated shoot^{-1} (Shah *et al.*, 2013). Each parameter was recorded on at least three biological replicates.

Statistical analysis

A completely randomized design was followed for the experiment. The data recorded for different parameters of surface sterilization, shoot regeneration, rhizogenesis was subjected to analysis of variance (Cochran and Cox, 1963).

RESULTS AND DISCUSSION

Surface sterilization of explants

P. orientalis explants (nodal and internodal segments) were subjected to 8 different sterilization regimes followed by culturing on MS basal medium. The effect of various sterilization regimes on explants survival, mortality and contamination percentages was highly significant (Table 1). The highest mean survival of 46.0% was observed in explants treated with 0.1% $HgCl_2$ for 10 min, followed by 40.0% in the explants treated with 0.1% $HgCl_2$ for 5 min. The response of internodal explants was significantly better (36.0%) than nodal explants (30.0%). Maximum percentage of surviving explants (51.9%) was when internodal explants were treated with 0.1% $HgCl_2$ for 10 min.

Maximum mean mortality of 44.8% was observed in explants treated with 0.3% $HgCl_2$ for 3 min followed by 40.3% in explants treated with 0.3% $HgCl_2$ for 2 min. The percent mortality of internodal explants was significantly lower (33.7%) than nodal explants (38.3%). The least mortality of 21.5% was observed in internodal explants treated with 0.1% $HgCl_2$ for 10 min. Maximum mean contamination of 34.1% was observed in explants treated with 0.3% $HgCl_2$ for 3 min. The lowest mean contamination of 26% was observed in explants treated with 0.1% $HgCl_2$ for 10 min. The contamination of internodal explants was significantly lower (30.6%) than nodal explants (31.7%). The maximum contamination of 35.9% was noticed in nodal explants treated with 0.3% $HgCl_2$ for 3 min and minimum contamination of 23.2% in internodal explants treated with 0.1% $HgCl_2$ for 1 min. The results are in close conformity with those obtained in *P. acerifolia* micropropagation through mesophyll protoplast culture (Liu *et al.*, 2002). They reported that the treatment of explants with 0.1% $HgCl_2$ for 10 min was effective for surface sterilization with maximum explant survival. Liu and Bao (2003) who worked on micro-propagation of *P. acerifolia* through adventitious shoot regeneration from *in vitro* cultured leaves found that the treatment of explants with 0.1% $HgCl_2$ for 10 min was the most effective surface sterilization procedure for maximum survival of explants with minimum tissue injury.

Shoot regeneration

Eighteen combinations of benzyl adenine, indole butyric acid, naphthalene acetic acid (BA + IBA or BA + NAA) in MS medium were assessed to evaluate their effects on shoot regeneration percent, number of shoots explant^{-1} and shoot length of nodal and intermodal shoot regeneration. Shoot regeneration response of internodal explants was significantly better than the nodal explants, which showed that the morphogenetic response of different explants, even from the same plant may

Table 1: Effect of concentration and duration of mercuric chloride sterilization of nodal and internodal explants of *Platanus orientalis* on explant survival, mortality and contamination

$HgCl_2$ treatment conc.& duration	Survival (%)			Mortality (%)			Contamination (%)		
	Nodal segments	Internodal segments	Mean	Nodal segments	Internodal segments	Mean	Nodal segments	Internodal segments	Mean
0.1% for 1 min.	30.8 (5.74)	37.3 (6.10)	34.0 (5.83)^{c}	36.5 (6.04)	39.5 (6.28)	38.0 (6.16)^{b}	32.7 (5.71)	23.2 (4.81)	28.0 (5.28)^{c}
0.1% for 2 min.	34.6 (5.88)	34.2 (5.84)	34.4 (5.86)^{bc}	32.7 (5.71)	31.5 (5.61)	32.1 (5.66)^{c}	32.7 (5.71)	34.4 (5.86)	33.6 (5.78)^{ab}
0.1% for 3 min.	26.9 (5.18)	34.6 (5.88)	30.7 (5.54)^{cd}	43.5 (6.59)	33.1 (5.74)	38.3 (6.18)^{b}	29.6 (5.44)	32.3 (5.68)	30.9 (5.56)^{b}
0.1% for 5min.	35.9 (5.98)	44.1 (6.63)	40.0 (6.32)^{ab}	31.6 (5.61)	23.3 (4.82)	27.5 (5.23)^{d}	32.6 (5.70)	32.7 (5.71)	32.7 (5.71)^{ab}
0.2% for 3 min.	24.9 (4.99)	29.7 (5.44)	27.3 (5.22)^{d}	41.3 (6.42)	39.3 (6.27)	40.3 (6.34)^{ab}	33.7 (5.80)	31.0 (5.56)	32.4 (5.68)^{ab}
0.3% for 2 min.	27.9 (5.28)	30.3 (5.50)	29.1 (5.39)^{cd}	41.1 (6.41)	37.6 (6.12)	39.4 (6.27)^{b}	31.0 (5.56)	32.1 (5.66)	31.6 (5.61)^{ab}
0.3% for 3 min.	18.9 (4.35)	25.6 (5.06)	22.3 (4.72)^{e}	45.2 (6.72)	44.3 (6.65)	44.8 (6.68)^{a}	35.9 (5.98)	32.4 (5.69)	34.1 (5.84)^{a}
0.1% for 10 min.	40.2 (6.34)	51.9 (7.20)	46.0 (6.78)^{a}	34.4 (5.86)	21.5 (4.63)	27.9 (5.28)^{d}	25.4 (5.03)	26.7 (5.16)	26.0 (5.10)^{c}
Mean	30.0 (5.47)^{B}	36.0 (5.99)^{A}		38.3 (6.18)^{A}	33.7 (5.80)^{B}		31.7 (5.63)^{A}	30.6 (5.52)^{B}	
$CD_{0.05}$									
Sterilant		0.49			0.37			0.24	
Explant		0.34			0.28			0.09	
Sterilant x explant		ns			ns			0.34	

Values in the parenthesis are square root transformed

vary. The variation in regeneration potential of different explants of same plants is well documented in many other plant species (Husaini *et al.* 2011). The interaction effect of different media and explants on shoot regeneration was significant with highest shoot regeneration (100%) observed in internodal explants cultured on MS medium supplemented with BA (4.00 mg L^{-1}) and IBA (0.25 mg L^{-1}). Least shoot regeneration of 13.2% was noticed in nodal segment explants cultured on MS medium supplemented with 1 mg BA L^{-1} + 0.5 mg IBA L^{-1}. The interaction effect of different media and explants on number of shoots explant^{-1} was significant with maximum number of shoots (9.7 explant^{-1}) in internodal explants cultured on MS medium supplemented with BA (4.00 mg L^{-1}) + IBA (0.25 mg L^{-1}). Least number of shoots (2.7 explant^{-1}) was obtained from the nodal segment explants cultured on MS medium supplemented with BA (1 mg L^{-1}) + IBA (0.25/0.5 mg L^{-1}). The interaction effect of different media and explants on length of shoots was significant. Maximum shoot length of 3.9 cm was observed in internodal explants cultured on MS medium supplemented with BA (4.00 mg L^{-1}) + IBA (0.25 mg L^{-1}) and minimum shoot length of 1.1 cm in nodal segment explants cultured on MS medium supplemented with BA (1 mg L^{-1}) + IBA (0.5 mg L^{-1}). The overall best shoot regeneration medium was MS medium supplemented with BA (4.0 mg L^{-1}) and IBA (0.25 mg L^{-1}) which showed maximum shoot regeneration percentage (72.8%), number of shoots explant^{-1} (7.5) and shoot length (3.0 cm) [Table 2]. The results are in consonance with Liu *et al.* (2002) who found IBA (0.25 mg L^{-1}) + BA (4.0 mg L^{-1}) best for shoot proliferation, number of shoots and maximum shoot length. Likewise, Liu and Bao (2003) reported maximum shoot proliferation percentage on 0.25 mg L^{-1} IBA and 4 mg L^{-1} BA.

Rhizogenesis

Shoots were rooted simultaneously on the same medium used for shoot regeneration, hence it is not necessary to subculture the shoots on separate rooting medium. Simultaneous development of

Table 2: Shoot regeneration from nodal and internodal explants of *Platanus orientalis* on MS medium supplemented with auxins and cytokinin, after 4 weeks

Growth regulators (mg L^{-1})			Shoot regeneration (%)		Mean	shoots explant^{-1} (No.)		Mean	Shoot length (cm)		Mean
NAA	IBA	BA	Nodal segments	Internodal segments		Nodal segments	Internodal segments		Nodal segments	Internodal segments	
0	0.25	1	13.4 (3.79)	23.4 (4.93)	18.4 (4.44)^{f}	3.7	4.7	4.2^{de}	1.4	2.0	1.7^{d}
0	0.5	1	13.2 (3.75)	20.0 (4.56)	16.6 (4.15)^{r}	2.7	4.3	3.5^{e}	1.1	1.4	1.3e
0	0.25	2	14.8 (3.97)	31.1 (5.66)	23.0 (4.81)^{e}	2.7	4.3	3.5^{e}	1.3	1.9	1.6^{d}
0	0.5	2	14.1 (3.88)	33.3 (5.85)	23.7 (5.01)^{e}	4.0	5.3	4.7^{cd}	1.4	1.8	1.6^{d}
0	1	2	14.8 (3.97)	33.3 (5.85)	24.1 (5.06)^{e}	4.0	5.3	4.7^{cd}	1.1	2.1	1.6 ^{d}
0	0.25	4	45.7 (6.82)	100 (10.05)	72.8 (7.79)^{a}	5.3	9.7	7.5^{a}	2.2	3.9	3.0^{a}
0	0.5	4	29.7 (5.54)	66.6 (8.21)	48.2 (6.73)^{b}	5.0	7.3	6.2 ^{b}	2.0	2.4	2.2^{b}
0	1	4	19.2 (4.49)	35.5 (6.03)	27.3 (5.11)^{e}	4.0	7.3	5.7^{bc}	1.9	2.0	1.9^{c}
0.25	0	1	20.7 (4.65)	29.7 (5.54)	25.2 (5.09)^{e}	4.0	6.7	5.3^{bc}	1.2	2.2	1.7^{d}
0.25	0	4	16.8 (4.21)	57.1 (7.61)	36.9 (6.01)^{c}	4.7	7.3	5.6^{b}	1.6	2.3	1.9c
0.5	0	4	25.0 (5.08)	50.0 (7.14)	37.5 (6.10)^{c}	3.3	7.3	5.3^{bc}	1.2	2.3	1.7^{d}
1	0	4	25.0 (5.08)	33.3 (5.85)	29.6 (5.51)^{d}	4.3	7.3	5.8^{b}	1.8	2.1	1.9^{c}
Mean			21.0 (4.59)^{B}	42.8 (6.44)^{B}		4.0^{B}	6.4^{A}		1.5^{B}	2.2^{A}	
$CD_{0.05}$											
Medium				0.30			1.06			0.15	
Explant				0.52			2.15			0.32	
Medium x Explant				0.83			ns			0.48	

Values in the parenthesis are square root transformed

shoots and roots, without transfer into new rooting medium, has been reported in other plant species (Husaini *et al.*, 2008). The number of roots with their length and regeneration per cent was calculated on the same culture media (Table 3). The interaction effect of different media and explants on root regeneration was significant. Highest root regeneration of 100% was observed

Table 3: Rhizogenesis of shoots of *Platanus orientalis* on MS medium supplemented with auxins and cytokinin, after 4 weeks

Growth regulators (mg L^{-1})			Root regeneration (%)		Mean	No. of roots/shoot		Mean	Root length (cm)		Mean
NAA	IBA	BA	Nodal segments	Internodal segments		Nodal segments	Internodal segments		Nodal segments	Internodal segments	
0	0.25	1	96.2 (9.85)	99.8 (10.03)	98.0 (9.94)^{ab}	4.7	7.3	6.0^{cd}	2.1	3.2	2.7^{b}
0	0.5	1	98.3 (9.96)	100 (10.05)	99.2 (10.00)^{ab}	9.3	12.0	10.7^{b}	2.1	3.2	2.7^{ab}
0	0.25	2	93.1 (9.69)	99.6 (10.03)	96.3 (9.86)^{cd}	4.0	7.3	5.7 ^{cd}	2.0	3.2	2.6^{b}
0	0.5	2	94.7 (9.77)	98.7 (9.98)	96.7 (9.87)^{cd}	4.0	7.3	5.7^{cd}	2.1	3.1	2.6^{b}
0	1	2	100 (10.05)	100 (10.05)	100 (10.05)^{a}	10.0	15.7	12.8^{a}	2.2	3.7	3.0^{a}
0	0.25	4	82.7 (9.14)	96.3 (9.86)	89.5 (9.50)^{e}	2.7	4.3	3.5^{e}	1.3	1.9	1.6^{d}
0	0.5	4	79.3 (8.96)	83.3 (9.28)	81.3 (9.12)^{f}	2.7	4.3	3.5^{e}	1.9	1.5	1.7^{cd}
0	1	4	91.4 (9.61)	98.6 (9.97)	95.0 (9.79)^{cd}	3.3	7.3	5.3^{d}	1.9	2.0	2.0^{c}
0.25	0	1	95.7 (9.83)	99.2 (10.01)	97.5 (9.92)^{bc}	4.3	9.7	6.7^{c}	2.2	3.4	2.8^{ab}
0.25	0	4	88.2 (9.44)	99.1 (10.20)	93.6 (9.82)^{cd}	4.0	6.0	5.0^{de}	2.0	1.8	1.9^{cd}
0.5	0	4	95.1 (9.80)	96.7 (9.88)	95.9 (9.84)^{cd}	3.7	4.7	4.2^{de}	2.0	1.8	1.9^{c}
1	0	4	92.6 (9.67)	96.8 (9.81)	94.7 (9.74)^{d}	3.3	7.3	5.3^{de}	2.0	3.0	2.5^{b}
Mean			92.3 (9.64)^{B}	97.3 (9.91)^{A}		4.7^{B}	7.7^{A}		2.0^{B}	2.7^{A}	
$CD_{0.05}$											
Medium				0.13			1.54			0.32	
Explant				0.20			2.13			0.51	
Medium x Explant				0.33			3.68			0.83	

Values in the parenthesis are square root transformed

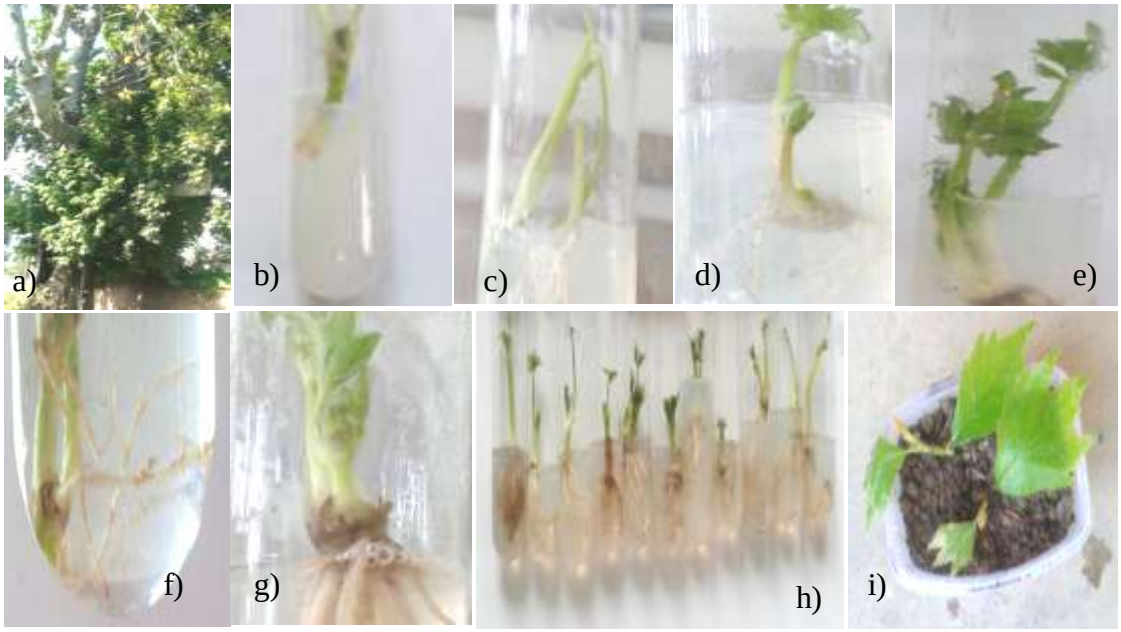

Fig. 1. *In vitro* propagation of chinar (*Platanus orientalis* L.): a) source of explants, the mother plant; b) explant on MS medium; c) shoot initiation; d) shoot elongation e) multiple shoot formation; f) root initiation; g) multiple root formation; h) complete plantlets; i) hardening in pots

when both nodal and internodal explants were cultured on MS medium supplemented with 2.0 mg L^{-1} BA + 1.0 mg L^{-1} IBA. The lowest root regeneration of 79.3% was obtained from nodal segment explants cultured on MS medium supplemented with 4.0 BA mg L^{-1} + 0.5 mg IBA L^{-1}. The interaction effect of different media and explants on number of roots per shoot showed highest number of roots 15.7 $shoot^{-1}$ when internodal explants were cultured on MS medium supplemented with BA (2.0 mg L^{-1}) + IBA (1.0 mg L^{-1}). Lowest number of roots 2.7 $shoot^{-1}$ was obtained from nodal segment explants cultured on MS medium supplemented with either 4.0 mg BA L^{-1} + 0.25 IBA mg L^{-1} or 4.0 BA mg L^{-1} + 0.5 mg IBA L^{-1}. The interaction effect of different media and explants on length of roots was significant. Maximum root length of 3.7 cm was observed in internodal explants cultured on MS medium supplemented with 2.0 BA mg L^{-1} + 1 mg IBA L^{-1} and a minimum of 1.3 cm from nodal segment explants cultured on MS medium supplemented with 4.0 BA mg L^{-1}) + 0.25 IBA mg L^{-1}. The overall best root regeneration medium was MS medium supplemented with 2.0 mg BA L^{-1} +1.0 mg IBA L^{-1} which showed maximum root regeneration percentage (100%), number of roots $shoot^{-1}$ (10), and root length (3 cm) [Table 3]. Reports of Gjuleva and Atanasov (1994) and Zencirkiran and Erken (2012) support our finding as they too obtained rooting with MS medium supplemented with IBA, BA, NAA from 0-4 mg L^{-1}. Liu *et al.* (2002) reported that 1.0 mg IBA L^{-1} + 2.0 BA mg L^{-1} was best for root proliferation, number of roots and highest root length while Guoqiang *et al.* (2003) reported that 1/2 MS + 0.1-0.5 mg IBA L^{-1} in medium was good for root induction. The complete *in vitro* protocol for regeneration of *P. orientalis* plants was developed which takes only 4 months to develop disease free plantlets of Chinar from these explants (Fig. 1).

Conclusions: Chinar (*Platanus orientalis* L.) is the only species of family Platanaceae found in India and has economic and cultural importance. The study developed first protocol for *in vitro* propagation of chinar by using nodes and internodes as explant materials. Various sterilization treatments yielded aseptic cultures but sterilization with 0.1% $HgCl_2$ for 10 min yielded maximum percentage of aseptic cultures and highest percentage of surviving explants. The survival response of internodal explants was significantly higher than that of nodal explants. The constituents of

medium especially growth regulators significantly influenced the regeneration potential of explants. MS medium with B_5 vitamins supplemented with IBA (0.25 mg L^{-1}) or BA (4 mg L^{-1}) was best for shoot regeneration; while MS medium supplemented with IBA (1 mg L^{-1}) o BA (2 mg L^{-1}) was most suitable for rhizogenesis. The shooting as well as rooting response of intermodal explants was significantly higher than nodal explants. The study shall be helpful in designing a comprehensive commercial tissue culture programme for chinar.

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REFERENCES

Anonymous. 2003. *Seed Propagation of Mediterranean Trees and Shrubs*. APAT/IGER, Rome, Italy.

Cochran, W.G. and Cox, G.M. 1963. *Experimental Designs* (3rd edn.). Asia Publishing House, Mumbai, India.

Dirr, A.M. and Heuser, C.W. Jr. 2006. *The Reference Manual of Woody Plant Propagation: From Seed to Tissue Culture*. Varsity Press Inc., North Carolina, USA.

Gjuleva, V. and Atanasov, A. 1994. Micropropagation of *Platanus acerifolia in vitro. Silvae Genetica*, **43**: 215-218.

Grolli, P.R., Morini, S. and Loreti, F. 2005. Propagation of *Platanus acerifolia* Willd. by cutting. *Journal of Horticulture Science and Biotechnology*, **80**: 705-710.

Guoqiang, F., Jianping, J., Yaoqing, H. and Feng, L. 2003. *In vitro* efficient plant regeneration with *Platanus orientalis* L. leaves as explants. *Acta Horticulturae Sinica*, **30:** 236-238.

Hartman, H.T., Kester, D.E., Davies, F.T. and Geneve, R.L. 2011. *Hartmann and Kester's Plant Propagation: Principles and Practices* (8th edn.). Prentice Hall, New Jersey, USA.

Husaini, A.M. and Abdin, M.Z. 2007. Interactive effect of light, temperature and TDZ on the regeneration potential of leaf discs of *Fragaria x ananassa* Duch. *In vitro Cellular and Developmental Biology-Plant* **43**: 576-584.

Husaini, A.M., Aquil, S., Bhat, M., Qadri, T., Kamaluddin and Abdin, M.Z. 2008. A high-efficiency direct somatic embryogenesis system for strawberry (*Fragaria*x *ananassa* Duch.) cultivar Chandler. *Journal of Crop Science and Biotechnology*, **11**: 107-110.

Husaini, A.M., Mercado, J.A., Schaart, J.G., Teixeira da Silva, J.A. 2011. Review of Factors Affecting Organogenesis, Somatic Embryogenesis and *Agrobacterium tumefaciens*-Mediated Transformation of Strawberry. In: Husaini AM and Mercado JA (Eds) *Genomics, Transgenics, Molecular Breeding and Biotechnology of Strawberry.* Global Science Books, UK, pp. 1-11.

Khosrojerdi, I., Tabari, M., Hosseini, M. and Rahmani, A. 2006. Diameter and number of cutting bud impact on sprouting of *Platanus orientalis. Journal of Agriculture Science and Natural Resources*, **12**(6): 189-194.

Kozgar, I. and Khan, S. 2011. Fiery chinar. *Science Reporter*, **48**(2): 54-55.

Liu, G. and Bao, M. 2003. Adventitious shoot regeneration from *in vitro* cultured leaves of London Plane tree (*Platanus acerifolia* Willd.). *Plant Cell Report*, **21**: 640-644.

Liu, G., Huang, J., Chen, L. and Bao, M. 2002. Plant regeneration from excised hypocotyl explants of *Platanus acerifolia* Willd. *In vitro Cell Developmental Biology-Plant*, **38**: 558-563.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia. Plantarum*, **15**: 473-497.

Pilloti, M., Brunneti, A., Tizzani, L. and Marrani, O. 2009. *Platanus acerifolia* genotypes surviving to inoculation with *Ceratocystis platani* (the agent of cancer stain): First screening and molecular characterization. *Euphytica*,**169**: 1-17.

Sert, S., Kutahyali, C., Inan, S., Talip, Z., Cetinkaya, B. and Eral, M. 2008. Biosorption of lanthanum and cerium from aqueous solutions by *Platanus orientalis* leaf powder. *Hydrometallurgy*, **90**: 13-18.

Shah, S.N., Husaini, A.M., Ansari, S.A. 2013. Micropropgation of *Litsea glutinosa* (Lour) C.B. *International Journal for Biotechnology and Molecular Biology Research* 4(5): 78-85

Sharma, S. 2008. *India - A Travel Guide*. Diamond Pocket Books, New Delhi, India.

Wadoo, M. 2007. *The Trees of Our Heritage*. Idris Publications, Kashmir, India. [*http://www. dnaindia.com/scitech/report-world-s-largest-chinar-tree-in-kashmir-1081483*).

Zencirkiran, M. 2010. Determination of native woody landscape plants in Bursa and Uludag. *African Journal of Biotechnology*, **8**: 5737-5746.

Zencirkiran, M. and Erken, K. 2012. The effect of different times collecting cutting and auxin treatments of the rooting in *Platanus orientalis*. *The Journal of Animal and Plant Sciences*, **22**: 764-767.

Evidence of proteoid roots in Chinar (*Platanus orientalis* L.): taxonomic implications

Oriental plane or Chinar (*Platanus orientalis*) is grown as an avenue tree in the Kashmir valley, India because of its canopy and autumn tint. It is a monoecious tree with encircling stipules, infrapetiolar buds, and usually palmately lobed and veined leaves. It carries pendant spike of heads bearing unisexual flowers and fruits of bristly achenes[1]. Though the tree tolerates a degree of drought, it prefers moist soils; it cannot grow in shade[2]. *Platanus* is the only extant genus belonging to the family Platanaceae that encompass eight known species[3]. While the family has traditionally been placed in the order Hamamelidales[4,5], recent phylogenetic studies suggest that it belongs to Proteales[6].

In the Kashmir valley, Chinar flowers during early spring, in March. Male inflorescences appear slightly earlier than the female ones. The globular assemblage of one-seeded achenes hangs on the tree until seed dispersal. Seeds mature in January of the following year, but seed dispersal starts in March. Though there are about 75,000 Chinar trees growing in the valley, natural regeneration is almost non-existent. Therefore, we examined whether the seeds were viable or not. If the seeds were viable, we explored further, whether they were dormant for the period between seed maturity (January) and seed dispersal (March).

While studying seed germination, we observed an interesting phenomenon: proteoid roots in the germinating seedlings (Figure 1). In light of this finding, we explore the relationship of Platanaceae with its sister family Proteaece.

For the study, we selected three sexually mature (diameter at breast height; dbh range: 179.8–320.1 cm), middle-aged trees growing in the Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Ganderbal, Kashmir. Seeds from these trees were collected twice, in January and March. The germination test was conducted immediately after seed collection. A replicated germination trial was laid out in a walk-in plant growth chamber (Blue Star; model no: BS-153) in January, immediately after the first seed collection. Nine hundred seeds from each of the three trees were selected randomly. A total of 2700 seeds were placed in petri dishes (27 in number), each containing 100 seeds on moist germination paper. We placed these in the growth chamber at 25° ± 2°C. The same procedure was followed for seeds collected in March.

We recorded germination count daily for one month. Seeds germination started from the fifth day onwards. Seedlings continued to emerge till the 18th day (Table 1). On an average, about 60% seeds germinated (Table 1). It shows that the seeds were viable and had good germination potential. Germination was slightly higher in the January seed-lot than in the March seed-lot. However, the difference was statistically non-significant ($P = 0.581$). This implies that the seeds were ready for germination as soon as they matured, thus indicating the absence of seed dormancy.

During the emergence of radicals, we noticed white filamentous growth covering it (Figure 1). Initially, it appeared as some fungal growth, but soon we identified these as proteoid roots. These roots were ephemeral and persisted on the radicals only for 8–10 days (Table 1).

Proteoid roots, also known as cluster roots, form clusters of closely spaced short lateral rootlets. Engler first noticed such roots in 1894 in the plants of the family Proteaceae. Purnell coined the term 'proteoid root'. She examined 44 species from ten Proteaceae genera (order: Proteales) and found proteoid roots in every genus, except *Persoonia*[7]. So far, proteoid roots are reported in about 1800 species belonging to ten families[8] (figure 4 of Lambers *et al.*[9]).

Proteoid roots have not been reported in any other species of Plantaceae so far. Even while growing over a year in low phosphorus nutrient solution, *Platanus hybrida* never produced proteoid roots[9]. They have not been reported in any other family of Proteales, except Proteaceae[8,9]. Proteoid roots are ephemeral and physiologically active for a little more than a few weeks[10,11]. The density of rootlets produced per unit root axis is far greater in the Proteaceae than in the other families[9]. Proteoid roots provide Proteaceae a competitive advantage over non-proteoid rooted species[12]. These plants can grow in soils deficient in nutrients, especially phosphorus-deficient soils[7].

As these roots are adaptive features of the plants that grow in nutrient-deficient soils, this finding may help in the study of adaptive behaviour in *P. orientalis*. Proteoid roots increase the surface area by over 140 times, and soil volume explored 300 times compared to an equivalent non-proteoid root. This increases the exudation of carboxylates, phenolics, solubilization of minerals and organic nutrients, thereby enhancing the uptake

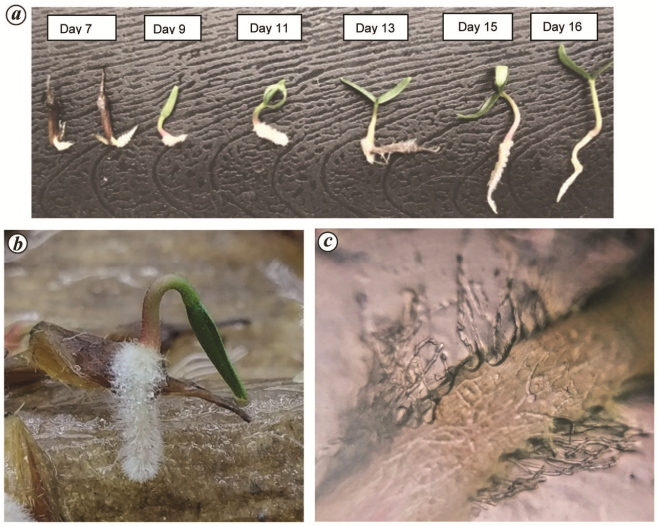

Figure 1. Proteoid roots in *Platanus orientalis*: (***a***) growth stages, (***b***) growth on 11th day after keeping the seed for germination and (***c***) longitudinal section (image magnification 400×).

Table 1. Seed germination and seedling emergence in *Platanus orientalis* in Kashmir, India

	Germination per cent		Germination progress		Duration of proteoid roots	
Seed source	January	March	Initiation	Termination	Appearance	Disappearance
Tree 1 (dbh: 179.8 cm)	59.67	68.33	7th day	18th day	7th day	15th day
Tree 2 (dbh: 202.4 cm)	63.33	44.33	6th day	17th day	6th day	15th day
Tree 3 (dbh: 320.1 cm)	61.67	61.33	5th day	18th day	6th day	16th day
Mean germination	61.56	58.00				
Overall mean	59.78					

of inorganic nutrients, amino acids and water per unit root mass[13,14]. These roots exude organic acids that solubilize phosphate associated with iron and aluminium in the rhizosphere[15]. The roots can help absorb inorganic phosphate (Pi) faster than non-proteoid roots[16,17]. In most species, clusters of proteoid roots decline as phosphorus availability to the roots increases, and when its supply is restricted, the clusters become prominent[18].

Presence of proteoid roots in *Platanus* has taxonomic implications as well. Before 1998 (pre-APG I phase), the Platanaceae was placed in the order Hamamelidales[4,5,19]. In 1998, it was transferred to Proteales[20]. Though all the subsequent APG classifications consistently placed Platanaceae in Proteales, the Thorne classification continued with the earlier scheme[21]. Finally in 2007, a revision of the Thorne classification accepted the change[22]. By 2009 (Angiosperm Phylogeny Group (APG) III), the position of Platanaceae got firmly established in Proteales[6]. Both these reports based their conclusions on molecular evidence. The molecular evidence based on gene sequences established the sister relationship of Platanaceae and Proteaceae[23,24]. The paucity of morphological evidence could be due to the ancient nature of the families. *Platanus* is considered a living fossil[25]. It is the only extant genus of the family Platanaceae[26], represented by as few as eight known extant species[3]. Though Doyle and Endress[27] mentioned orthotropous ovules, free nuclear endosperm development and large embryos as similarities between Platanaceae and Proteaceae, these were not sufficient to club them together in one order[20].

The molecular evidence for the closeness of the relationship between Platanaceae and Proteaceae has promoted the search for previously obscure morphological synapomorphies. Carpenter *et al.*[24] found similarity of trichome base structure in the two families. They interpreted it as another morphological similarity – the first non-reproductive trait – linking the two families. Our finding of proteoid roots in *P. orientalis* provides one more such evidence.

1. Simpson, M. G., *Plant Systematics*, Academic Press, Amsterdam, The Netherlands, 2010, pp. 275–448.
2. http://www.euforgen.org/species/platanus-orientalis/ (accessed on 16 October 2020).
3. Christenhusz, M. J. M. and Byng, J. W., *Phytotaxa*, 2016, **261**, 201–217.
4. Cronquist, A., *The Evolution and Classification of Flowering Plants* (*2nd edn*), New York Botanical Garden, New York, USA, 1988.
5. Takhtajan, A. L., *Diversity and Classification of Flowering Plants*, Columbia University Press, New York, USA, 1997.
6. Angiosperm Phylogeny Group III, *Bot. J. Linn. Soc.*, 2009, **161**, 105–121.
7. Purnell, H. M., *Aust. J. Bot.*, 1960, **8**, 38–50.
8. Lamont, B. B. and Pérez-Fernández, M., *Ann. Bot.*, 2016, **118**, 725–732.
9. Lambers, H., Shane M. W., Cramer M. D., Pearse, S. J. and Veneklaas, E. J., *Ann. Bot.*, 2006, **98**, 693–713.
10. Shane, M. W., Cramer, M. D., Cawthray, G. R., Kuo, J. and Lambers, H., In *Plant Nutrition for Food Security, Human Health and Environmental Protection* (eds Li, C. J. *et al.*), Tsinghua University Press, Beijing, China, 2005, pp. 522–523.
11. Playsted, C. W. S., Johnston, M. E., Ramage, C. M., Edwards, D. G. and Lambers, H., *New Phytol.*, 2006, **170**, 491–500.
12. Fajardo, A. and Piper, F. I., *Ann. Bot.*, 2019, **124**, 1121–1131.
13. Lamont, B. B., *Plant Soil*, 2003, **248**, 1–19.
14. Lambers, H., Martinoia, E. and Renton, M., *Curr. Opin. Plant. Biol.*, 2015, **25**, 23–31.
15. Zúñiga-Feest, A., Delgado, M. and Alberdi, M., *Plant Soil*, 2010, **334**, 113–121.
16. Vorster, P. and Jooste, J., *S. Afr. J. Bot.*, 1986, **52**, 277–281.
17. Hocking, P. J., Keerthisinghe, G., Smith, F. W. and Randall, P. J. A., In *Plant Nutrition for Sustainable Food Production and Environment* (eds Ando, P. *et al.*), Kluwer Academic Publishers, Tokyo, Japan, 1998, pp. 305–308.
18. Dinkelaker, B., Hengeler, C. and Marschner, H., *Acta Bot. Bras.*, 1995, **108**, 183–200.
19. Thorne, R. F., *Aliso*, 1992, **13**(2), 365–389.
20. Angiosperm Phylogeny Group (I), *Ann. Miss. Bot. Gard.*, 1998, 85(**4**), 531–553.
21. Thorne, R. F., *Bot. Rev.*, 2000, **66**(4), 441–647.
22. Thorne, R. F. and Reveal, J. L., *Bot. Rev.*, 2007, **73**(2), 67–181.
23. Wikstrom, N., Savolainen, V. and Chase, M. W., *Proc. R. Soc. London, Ser. B*, 2001, **268**, 2211–2220.
24. Carpenter, R. J., Hill, R. S. and Jordan, G. J., *Int. J. Plant Sci.*, 2005, **166**, 843–855.
25. Sanderson, M. J. and Doyle, J. A., *Am. J. Bot.*, 2001, **88**, 1499–1516.
26. Nixon, K. C. and Poole, J. M., *Lundellia*, 2003, **6**, 103–137.
27. Doyle, J. A. and Endress, P. K., *Int. J. Plant Sci.*, 2000, **161**, 121–153.

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An Efficient Method for Development of Callus and Suspension Culture in *Crocus Sativus.L* (Saffron) Using 6-BAP, 2-4 D and NAA Enriched Media

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Abstract:

Saffron (*Crocus sativus* L.) is a member of Iridaceae and is one of the most valuable spices in the world.The crop do not form seeds and reproduce by the budding of the existing corms. The corms are vulnerable to many pathogens and in order to produce healthy, disease free plants, pathogen free planting material is of great importance for successful cultivation of saffron. We compared the 2 methods for production of callus and suspension culture on MS media. We observed that the solid MS media containing the hormones with concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA produced high quality callus in 45-60 days. The liquid MS media containing the same composition of the hormones showed decent growth of cells in 14 days. This method can be employed to produce high quality callus and suspension culture for developing somatic embryos or study the gene expression of apocarotenoid genes and floral development genes in the cells and callus. The suspension culture can also provide starting material for induction of mutation in the saffron.

Introduction:

Saffron is one of the high value spice cultivated in several countries in the world including Iran, Spain, Greece and Kashmir (Fernandez, 2004). The flowers have six petals, three on the inner side and three on the outer side joined on the long pipe that comes out of the upper part of the ovary (Mir et al., 2012). The form is regular and straight. The column separated from the top of the underground ovary ends in a sole stigma of three threads that have an intense red color. The leaves, also called nomophyllus, vary in number from 10 to 15 for each shoot. Their color is dark green with a white middle stripe in the inner side and a venation on the outside (Nehvi et al., 2005). Pathogens affect the yield of this crop and remain active in corms after harvesting (1). Due to poor quality of planting material, the yield of saffron is low and to overcome this problem

saffron propagation via tissue culture may offer a boost for high production of pathogen free spice (Draget et al. 1988). Tissue culture offers a fast and reliable means to produce pathogen-free planting material for healthy plants. The callus and suspension culture cells have been used in many gene expression studies of apocarotenoid genes and floral development genes (Wafai et al., 2015). We have standardized the procedure for producing callus and suspension culture from corms of saffron using different concentrations of hormones on in MS media. Our method provided a good quality callus and suspension culture in short span of time.

Sample Collection:

Corms were collected directly from field and stored at room temperature. Extreme caution was taken while collecting the samples and only healthy corms were selected for tissue culture.

Establishment of callus culture and suspension culture:

Preparation of Ex-plant:

Fresh corms were collected from the field and washed vigorously with Sterilized water. All the fibers on the corms were removed and the white corm was washed with 1% Tween solution for 2 mins. Under the laminar flow, the corms were treated with 0.12% $HgCl_2$ for 2-3 minutes.The treated corms were washed 3 times for 1 minute with sterilized water. The corms were treated with 25 units/ml solution of Nystatin for 5 minutes. The corms were washed 3 times with sterilized water for 30 seconds. A corm was placed in a sterilized petri dish and the central portion of 8mmX8mm was cut using a fresh scalpel and same procedure was implemented with other corms too.

Media Preparation:

MS Agar Media was prepared which had following constituents: 0.44% MS media, 3% Sucrose, 0.8% Agar, Plant hormones (1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA). pH was adjusted to 5.6 using HCl. The MS agar media was autoclaved at 121^0C for 15 minutes under 15 psi pressure and poured into sterilized 500mL conical flask and sealed the mounted a cotton plug on the mouth of flask. The media was allowed to solidify at room temperature for 1 hour. Liquid MS media was prepared having similar composition as MS agar media except the agar. 50mL liquid MS media was put in a 200mL conical flask and sterilized in autoclave at 121^0C for 15 minutes at 15 psi pressure. The flask was cooled and placed at room temperature.

Callus Culture:

In a laminar flow hood, a piece of already treated corm was placeed on the surface of solid MS media in the flask using sterile forceps. The ex-plant was gently pushed so that $1/4^{th}$ of the ex-plant was embedded in the media. 5-6 ex-plants were placed in a 200mL conical flask and the mouth of the flask was closed with a cotton plug under the flame. The flask was incubated in an incubator at 22^0C with 8 hours of dark and 16 hours of light intervals for 45-60 days.

Suspension Culture:

The flask containing liquid MS media was placed in a laminar flow hood and opened near the flame. The callus on the MS agar media was put on sterilized petri dish and the actively growing part was cut off using a fresh scalpel and directly dropped into the liquid culture. The flask was incubated in a shaking incubator at 22^0C at 200 rpm with 8 hours dark and 16 hours light intervals for 12-15 days. After 15 days 5 ml culture media was added to fresh 45mL MS media and incubated at 22^0C at 200 rpm with 8 hours dark and 16 hours light intervals for 10-12 days. Sub-culturing was done after every 12-15 days in order to prevent cells from the stress due to accumulation of toxins and nutrient deprivation.

Result and Discussion:

After incubating the explants on MS media for 45-60 days with 16 hours light and 8 hours dark light cycles we observed a visible callus developing from the explants. The callus developed at two different concentrations of hormones, with 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA and 3.5mg/L 2,4-D, 0.5mg/L 6-BAP and 1.5mg/L NAA. After 45 days callus was visible in samples with both concentrations of hormones. However, after 60 days the flask with hormone concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA had good quality callus compared to the callus in the flask with hormone concentration of 3.5mg/L 2,4-D, 0.5mg/L 6-BAP and 1.5mg/L NAA. Therefore callus developed on media with hormone concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA was used for suspension culture development (Fig 1)

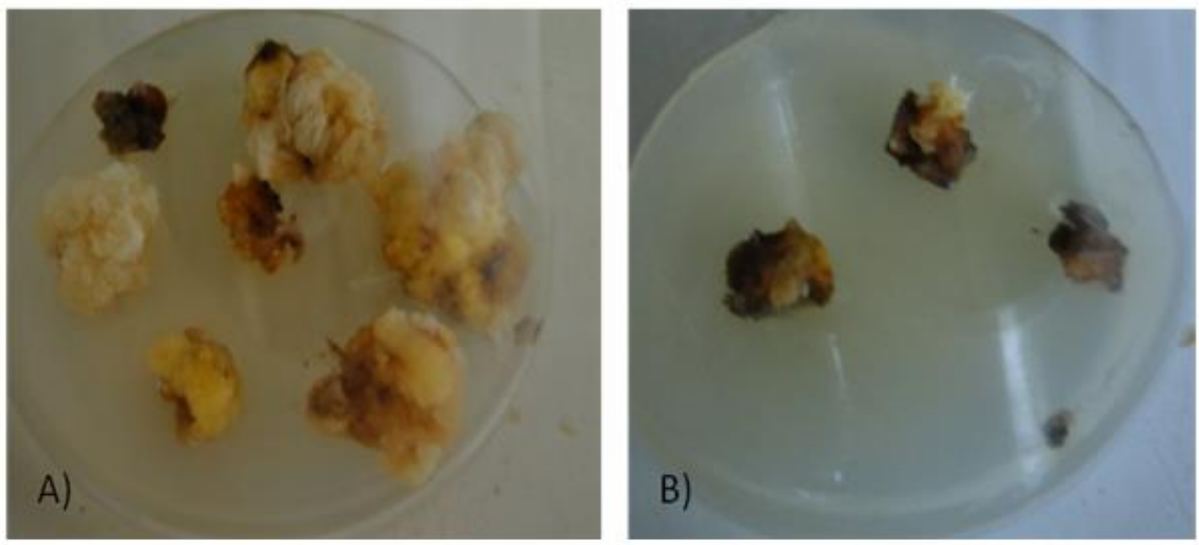

Fig 1: A) Callus developed on the media with the hormone concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA; B) Callus developed on the media with the hormone concentration of 3.5mg/L 2,4-D, 0.5mg/L 6-BAP and 1.5mg/L NAA.

The suspension culture was developed by inoculation of healthy callus fragments in meshed form to the liquid MS media with hormone concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA. The culture was incubated at 22^0C with rotation of 150 RPM and photoperiod of 16 hours light and 8 hours darkness. Sub-culturing the suspension culture after 20 days, we observed that the growth of cells was visible in liquid media as slight yellowish color started to appear due to accumulation of apocarotenoids (Fig. 2A).

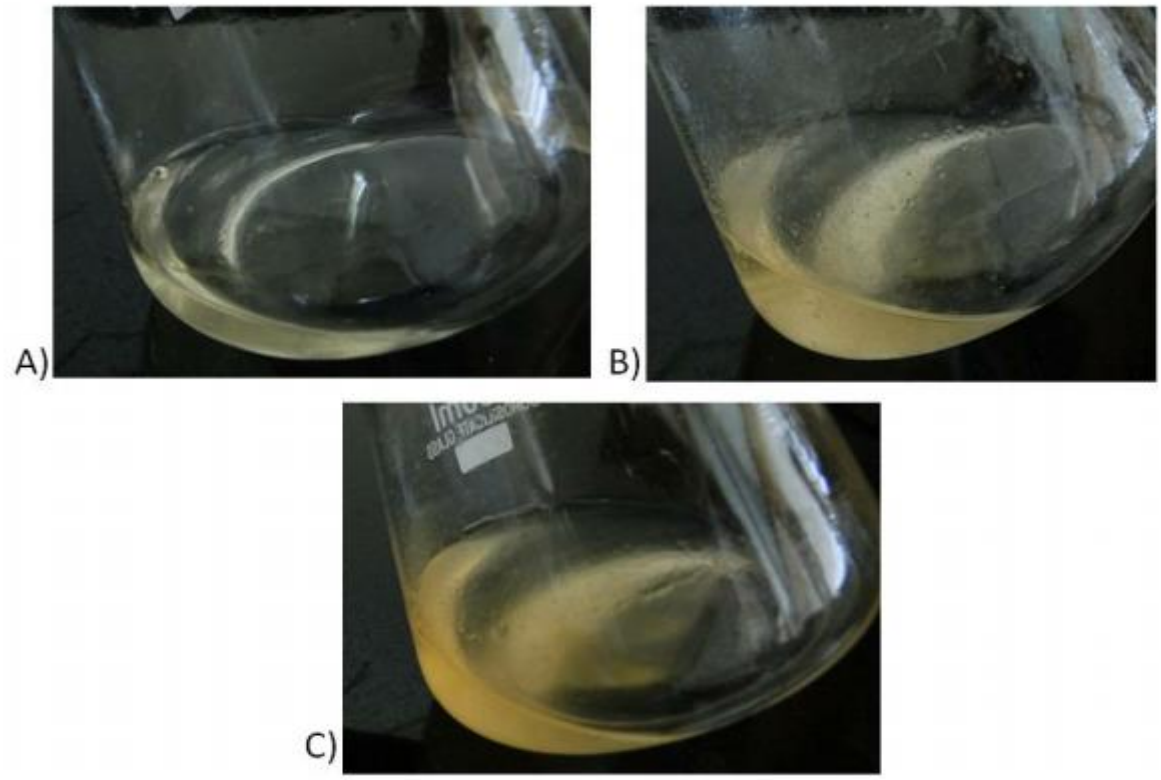

Fig 2: Suspension Culture after A) 7 days post inoculation; B) 14 days post inoculation; C) 25 days post inoculation.

After 14 days, the cell concentration in suspension culture was high. The color of the culture further transformed into deep yellow and the viscosity of the culture increased. (Fig.2B). The cell growth was observed to be maximum at this stage (log phase). However after 25 days, there was visibly high concentration of apocarotenoids (Fig 2C). It was observed that the cells harvested after 14 days of sub-culturing were best suited for sub-culturing and other assays.

Conclusion:

We conclude that the concentration of 1.5mg/L 2,4-D, 1.5mg/L 6-BAP and 0.5mg/L NAA in MS media produced good quality callus and suspension culture. It was observed that callus was developed in 45 days post inoculation. The suspension culture developed from callus in 12-15 days post inoculation. The cells from suspension culture can be used to produce somatic embryos or artificial seed. The efficient production of suspension culture can pave way for efficient micro propagation of saffron plant.

References:

1. Nehvi F A, Agarwal S G, Mir M A, Dar S A, Mir Z A and Nusrat N. Quality drying of saffron. 2005. SKUAST J. Res. 7: 343–346.
2. Mir J I, Ahmed N, Wafai A H & Qadri R A. Relative expression of CsZCD gene and apocarotenoid biosynthesis during stigma development in Crocus sativus L. 2012. Physiol Mol Biol Plants 18(4):371–375.
3. Wafai A H, Bukhari S, Mokhdomi T A, Amin A, Wani Z, Hussaini A, Mir J I & Qadri R A. Comparative expression analysis of senescence gene CsNAP and B-class floral development gene CsAP3 during different stages of flower development in Saffron (Crocus sativus L). 2015. Phy & Mol Bio of Plants. 21(3):459-463.
4. Fernandez J A. Biology, biotechnology and biomedicine of saffron. 2004. Recent Res. Dev. Plant Sci. 2:127–159.
5. Vahedi M, Kalantari S and Salami S A. Factors Affecting Callus Induction and Organogenesis in Saffron (Crocus sativus L.). 2014. Plant Tissue Cult. & Biotech. 24(1): 1-9.
6. Debergh PC and Read PE. Micropropagation. In: Micropropagation Technology and Applications. 1991. Debergh, PC and Zimmerman, RH (Eds.) Kluwer Acad. Pub. Dordrecht. pp. 1-13.