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MICROPROPAGATION AND CALLOGENESIS OF *PISUM SATIVUM* L. CULTIVARS, CLIMAX AND JANASS

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Abstract

To produce the disease free plants, technique developed and known parallel to conventional breeding is plant tissue culture (PTC). In present studies, culture conditions were optimized for micropropagation and callogenesis of Pea (*Pisum sativum* L**.)** cultivars (Climax and Janass) from different explants in MS medium added with different plant growth regulators (PGRs). MS medium (fortified with 5+0.5mg/L of BAP+NAA) showed 85% shoot initiation response from nodal explants of cv. Jannas. There were 4 shoots and 4 leaves per shoot on average per culture. While in case of cv. Climax, 92% induction from the same explant was obtained with a lower concentration of BAP+NAA (3.0+0.5) mg/L. The culture showed 3 shoots and 5 leaves per culture. Rooting was also visible in all cultures of shoots grown in MS basal medium. Jannas cultivar showed 65% of callus response after 20 days of inoculation in MS medium supplemented with 2,4-D+BAP (3.0+0.5mg/L). Callus formed was and green whereas callus response (70%) of climax cultivar was observed after 18 days of culturing in MS medium fortified with 2,4-D+BAP (2+0.5mg/L) and callus formed was green and granular.

Key Words: Green Technology, Nano Biotechnology, Nanoparticles, Natural Product, Secondary Metabolites**.**

Introduction

Pisum sativum L. (Pea) is an important crop plant belonging to subfamily Papilionaceae. It is considered to be one of the oldest cultivated crops and is likely to be originated from Southwest Asia. Pea contains about 5.4% of total protein content along with many minerals, like potassium, iron, magnesium, zinc, manganese, and phosphorus in significant amounts and many other vitamins. It also contains antioxidants including, Alpha Lipoic Acid, Glutathione, Lutein and Zeaxanthin (Ghanem *et al*., 1996; Das *et al*., 2014) and phytonutrients called Pisumsaponins I and II, and Pisomosides A and B (Rickman *et al.*, 2007).

Different cultivars of pea are cultivated in different areas of Pakistan for their freshness and taste. Peas are cool-season crops and are grown in early spring or late summer in well- drained soils. Growth of pea plant is affected by a number of viral and fungal diseases. Fungal diseases include damping off, root rot, downy and powdery mildew, Aschochyta blight and *Fusarium* wilt (Weeden, 2007; Grunwald *et al*., 2004). Similarly, there are many other reasons which are also responsible for reduction of yield like poor cultivar, insects, pests, poor management policies and temperature effects etc. Pea production can be enhanced by considering the diverse ecological conditions and irrigation schemes. Due to an increase in market demand of peathere is need to use improved field technologies to grow and irrigate pea fields in proper way. It is also required to produce disease free plants to satisfy the demand of end users (Ratnadas *et al*., 2012).

Plant tissue culture is an alternative to conventional breeding program. It's a rapid type of technique which can produce required number of plants throughout the year in controlled environment of tissue culture lab. It is an alternative of true breeding of plants which used different parts of plants as explants (as a source of tissue culture) like shoot tips and nodal explants. It's an economical technique in terms that it can produce a large number of disease free plants and their cultures can be maintained in small areas of laboratory (Butt *et al*., 2015; Shahzad *et al*., 2017). There are earlier reports on success of tissue culture for producing disease free plants. Jackobson and Hobb (1990) reported the production of multiple shoots of pea plants from cotyledonary node explants. Griga and Stejskal (1994) observed the multiple shoots in pea plants when MS medium fortified with 20uM BAP and 0.1 uM NAA and meristems and buds were used as explant.

Callus is a collection of undifferentiated cells which can be used to produce a number of plants. It's another useful technique of plant propagation in PTC. Callus cultures can be produced by optimizing the culture parameters like hormonal doses, light and type of nutrients (Bourgaud *et al.,* 2001). Another good feature of callus is its genetic variability which makes it a source of different selectable plants. It is the reason that plants with new genetic makeup can be produced and maintained. Hussey and Gunn (1984) reported the production of callus from pea explants which were inoculated on MS medium (Murashige and Skoog, 1962)+ BAP (1.0 mg/L) + IBA (4-8mg/L). Explants used were pea plumules and callus initiated from base of plumules. Ghanem *et al. (*1996) used MS+2,4-D+KN to get the callus response of pea explants. They used different doses of plant growth regulators for different explants. Manjubala *et al.* (2010) reported the callus formation on MS+2,4-D (5ppm) using leaf explants whereas El Sayed (2011) were successful to get the callus response of pea seedlings from MS +KN+2,4-D (0.2+2.0 mg/L).

The present study was aimed to get the callus cultures of two cultivars (i.e., Jannas and Climax) of *P. sativum* L. from sterilized *in vitro* grown explants. For this purpose different combinations and doses of plant growth regulators were optimized.

Materials and methods

The present work was carried out in Plant Biotechnology Research Laboratory, Dept. of Botany, University of the Punjab, Lahore, Pakistan. Two cultivars of pea plant (*Pisum sativm* L.) selected for the study were Climax and Janass. These cultivars were grown and developed in the Botanical Garden of the University of the Punjab, Lahore,

Pakistan.

Micropropagation: Apical meristem of grown cultivars was used as an explant. Apical meristems were cut finely from sterilized shoot tips. Shoot tips were sterilized using 0.1mg/L mercuric chloride. After washing with tap water, shoot tips were immersed in mercuric chloride solution for 3-5 minutes with continuous shaking on orbital shaker. These shoot tips were then washed with sterilized water under laminar air flow cabinet. After that those were inoculated on MS medium to get sterilized *in vitro* grown plants to be used as an explant for callus cultures. MS medium was used with different doses of Benzyl aminopurine (BAP) and Naphthalene acetic acid (NAA) for shooting cultures of both cultivars while for rooting basal MS medium (Phytoregulator free) was used.

Callogenesis: For callus induction, leaf explants were taken from *in vitro* grown plantlets and cultured on MS medium supplemented with different combinations of 2 , 4 - dichlorophenoxy acetic acid (2, 4-D) with BAP, and 2,4-D with Kinetin (KN). Well proliferating callus cultures were maintained by sub-culturing in the medium of same concentration of growth regulators after every 2 weeks and results were recorded.

Culture conditions: the culture conditions were set as light period of 16 hours and dark period of 8 hours. To maintain the light intensity between 2500-3000 lux, fluorescent lights of 40W were used. Temperature of culture room was set and maintained at 25º C.

Results

Micropropagation: Table 1 presents the effect of plant growth regulators on shooting response (shoot initiation and no. of shoots per explant) of both cultivars of pea plant. In cv. Climax, maximum shoot induction (57%) and number of shoots (4 /culture) were observed in MS medium containing 5.0 mg/L BAP (Plate 1a). The results were recorded after two weeks of inoculation. In cv. Janass, maximum shoot induction (67%) was obtained in MS medium containing 5.0 mg/L BAP with an average of 4 shoots per culture. While minimum (20%) shoot induction was observed in control and in MS medium supplemented with 1.0 mg/L BAP (Table 1, Plate 1b).

When a combination of BAP with NAA was tried in MS medium for shoot induction, cv. Climax showed 92% response in MS medium supplemented with BAP+NAA (3.0+0.5mg/L) with maximum shoot number 3/culture), while minimum response (40%) was observed in MS medium containing BAP+NAA(5.0+0.5mg/L) (Table 1, Plate 1c). In cv. Janass, shoot induction response ranged from 45 to 85% in different media combinations. Maximum result (85%) was observed in MS medium containing BAP+NAA (5.0+0.5mg/L) and average number of shoots were 4/culture (Table 1, Plate 1d).

Callogenesis: The callus induction was observed from leaf explants of pea cultivars after 20-25 days of inoculation, leaf explants of pea cultivars showed callusing response within 20-25 days. In climax cv. 65% and 70% callogenic response was shown by MS medium fortified with 2,4-D and KN (4.0+0.5mg/L) and 2,4-D and KN (5.0+0.5mg/L) respectively. The calli produced was green and granular (Plate 2a). Whereas only cultivars in MS medium supplemented with lower doses of 2,4-D +KN (1+0.5mg/L) showed lesser response, recorded as only 20% of cultures (Table 2).

Callus cultures of Jannas cv. also showed similar response where leaf explants when inoculated in MS medium fortified with 2,4-D and KN (5+0.5mg/L), showed 60% callogenic response; Whereas minimum callusing response was noticed in cultures with MS medium + 2 , 4 - D+ KN (1.0+0.5mg/L). Only 20% of these cultures showed callusing response after 20 days of inoculation (Table 2, Plate 2b).

In cv. Climax, maximum of 70% callus induction response was recorded in MS medium containing 2.0 mg/L of 2, 4-D with 0.5 mg/L BAP producing green and granular callus (Plate 2c). In MS medium having 1.0 mg/L 2, 4-D with 0.5 mg/L BAP, response to callus induction was 50%, while minimum (27%) callus formation was observed with 4.0 mg/L 2,4-D and 0.5 mg/L BAP (Table 2). In cv. Janass, maximum response to callus induction was exhibited by MS medium supplanted with 3.0 mg/L 2, 4-D with 0.5 mg/L BAP, which gave 65% response followed 50% response in medium having 2.0 mg/L 2,4-D with 0.5 mg/L BAP with globular and green calli (Table 2, Plate 2d).While minimum (10%) response to callus induction was observed in medium containing 5.0 mg/L 2, 4-D with 0.5 mg/L BAP in which the calli were yellowish green and globular.

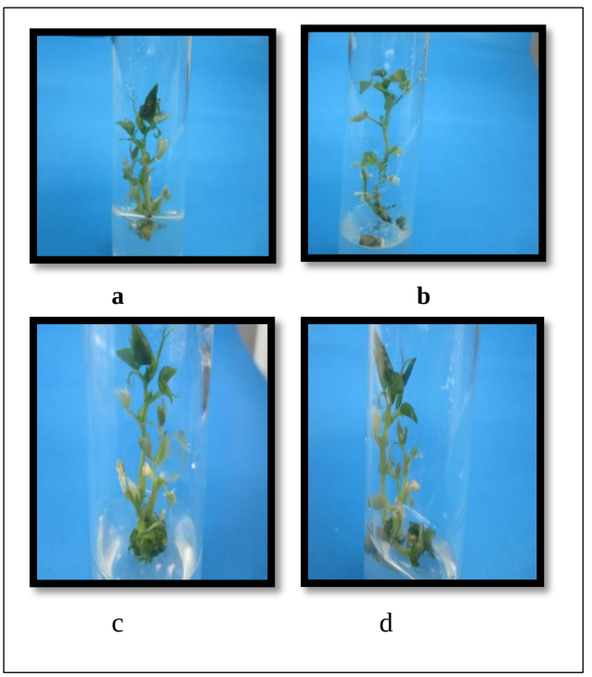

Plate 1: (a) Shoot response from nodal explants of *P. sativum* L. cv. Climax and (b) cv. Janass on MS medium supplemented with 5.0mg/L BAP, (c) shoot induction from nodal explants of cv. Climax in 3.0mg/L BAP with 0.5mg/L NAA and (d) shoot induction in cv. Janass in MS medium containing 5.0mg/L BAP with 0.5mg/L NAA.

Table 1: Effect of different PGRs on shoot response of *P. sativum* L. cvs. Climax and Janass

Treatment	Pea cultivar Climax			Pea cultivar Janass		
BAP (mg/L)	**Shoot induction (%)**	**Avg. no. of shoots per culture**	**Avg. no. of leaves per shoot**	**Shoot induction (%)**	**Avg. no. of shoots per culture**	**Avg. no. of leaves per shoot**
Control (MS)	10 ± 2.88 ^{cd}	2	2	20 ± 2.88 ^{bc}	2	2
MS + 1.0	23 ± 1.45 ^{c}	1	2	20 ± 2.88 ^{bc}	1	2
MS + 2.0	40 ± 2.88 ^{b}	2	2	30 ± 2.88 ^{b}	2	2
MS + 3.0	40 ± 2.88 ^{b}	3	2	30 ± 2.88 ^{b}	3	2
MS + 4.0	50 ± 2.88 ^{ab}	3	4	60 ± 2.88 ^{a}	3	4
MS + 5.0	57 ± 1.15 ^{a}	4	6	67 ± 1.45 ^{b}	4	6
BAP+NAA (mg/L)						
Control (MS)	10 ± 1.15^{d}	2	2	20 ± 2.88^{d}	1	2
MS + 1.0 + 0.5	42 ± 1.15^{c}	3	3	45 ± 2.88^{c}	1	3
MS + 2.0 + 0.5	62 ± 1.45^{bc}	3	3	62 ± 1.45^{bc}	1	3
MS + 3.0 + 0.5	92 ± 1.45^{a}	3	5	70 ± 2.33^{b}	1	3
MS + 4.0 + 0.5	75 ± 1.15^{b}	3	2	77 ± 1.15^{ab}	2	3
MS + 5.0 + 0.5	40 ± 1.15^{c}	2	2	85 ± 1.45^{a}	4	4

Table 2 Effect of 2,4-D with KN on callus induction from leaf explants of *P. sativum* L. cvs. Climax and Janass.

Treatment	Pea cultivar Climax		Pea cultivar Janass	
2,4-D + KN (mg/L)	**Callus induction (%)**	**Callus morphology**	**Callus induction (%)**	**Callus morphology**
Control	0	-	0	-
MS + 1.0 + 0.5	20 ± 2.88^{de}	Green, granular	20 ± 2.88^{cd}	Granular, green, friable
MS + 2.0 + 0.5	30 ± 2.88^{d}	Green, granular	30 ± 2.88^{c}	Globular, green, friable
MS + 3.0 + 0.5	50 ± 2.88^{c}	Yellowish green, compact	45 ± 2.88^{bc}	Light green, compact
MS + 4.0 + 0.5	65 ± 2.88^{ab}	Yellowish green, friable	50 ± 2.88^{b}	Light green, yellowish, compact
MS + 5.0 + 0.5	70 ± 2.88^{a}	green, granular	60 ± 2.88^{a}	Light green, granular
2,4-D + BAP (mg/L)				
Control	0	-	0	-
MS + 1.0 + 0.5	50± 2.88 ^{b}	Green, granular	45 ± 2.88^{bc}	Globular, green, compact
MS + 2.0 + 0.5	70 ± 0.66 ^{a}	Green, granular	50 ± 2.88^{b}	Globular, green, granular
MS + 3.0 + 0.5	40± 2.88 ^{c}	Globular green	65 ± 1.15^{a}	Yellowish green, granular
MS + 4.0 + 0.5	30± 2.88 ^{d}	Yellowish green callus	23 ± 0.88^{d}	Globular, Green
MS + 5.0 + 0.5	27 ± 2.88 ^{a}	Yellowish green	10 ± 2.88^{de}	Globular, green

The reported results are obtained from values of three replicates as mean values. Their significance is tested at 5% level of significance with ± SE. Values with different superscript letter show significant difference and vice versa

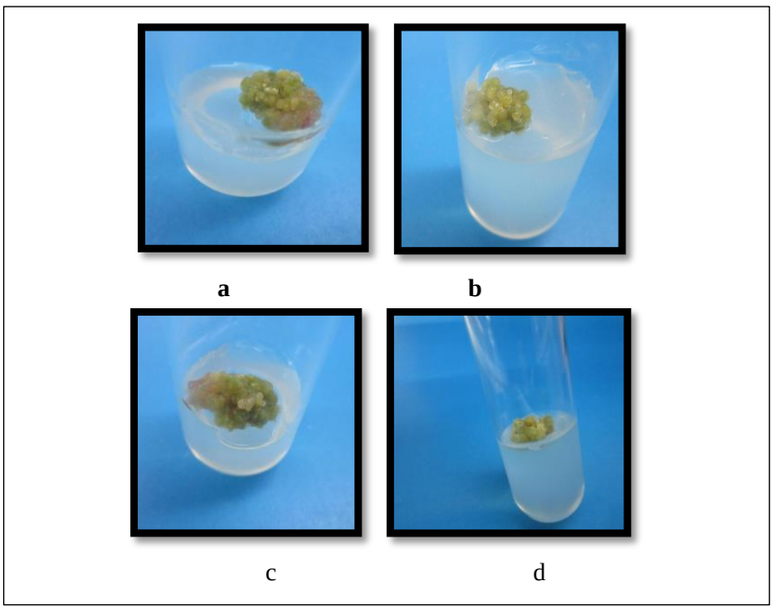

Plate 2: (a) Callus formation from leaf explants of *P. sativum* L. cv. Climax and (b) cv. Janass on MS medium supplemented with 5.0mg/L 2,4-D with 0.5mg/L KN, (c) Callus formation from leaf explants of cv. Climax in 2.0mg/L 2,4-D with 0.5mg/L KN and (d) callus formation in cv. Janass in MS medium containing 3.0mg/L2,4-D with 0.5mg/L KN.

Discussion

Micropropagation: shoots initiation and their multiplication in plant tissue culture media depend mainly upon two main factors i.e., type of explant used and dose of plant growth regulator. It is a common experience of getting shoot initiation response with cytokins in the culture medium while using shoot tips or nodal explants (Reddy *et al.*, 2014). Same response was observed in the present study where pea cultivars gave positive results with shoot initiation and multiplication from nodal explants. Cultures of both the cultivars i.e., Jannas and Climax, produced 67% and 57% shoot initiation responses in MS medium supplemented with BAP at a dose of 5mg/L.

Cytokinins like BAP have a long history of being used as shoot initiators in plant tissue culture experiments (Huang *et al*., 1994; Porika *et al.*, 2009; Timofeeva *et al.*, 2014). Reddy *et al.*, (2014), while working on *Musa sp.*, reported the positive response of explants of cornlets on MS medium with BAP (2.0 mg/L). Earlier Jackson and Hobb (1990) reported successful rapid shoot multiplication results of pea nodes on MS + BAP (1.0mg/L) medium. Pniewski *et al.* (2003) worked on several Polish pea cultivars and reported good shoot induction on MS+BAP 4.5mg/L.

To maximize the shooting response of pea cultivars, BAP and NAA were also used in combination in the current research work and results obtained were quite promising. A combination of higher dose of BAP with a lower dose of NAA gave an optimized result for shoot induction i.e., 85% for Climax cultivar and 92% Jannas cultivar. Similar response was reported by the Griga *et al.*, (1986) who used modified Murashige and Skoog medium. They used shoot apices as explants and supplemented medium with BAP and NAA and got optimized results with MS+BAP (20 uM)+NAA(0.1uM). Ghanem *et al.* (1996) used Egyptian variety of *Pisum sativum* L. They used hypocotyl of the pea seeds as an explant and reported best results with a combination of BAP and NAA (2.0+1.0 mg/L) BAP and NAA. In 2004, Kumar and Mathur, published their work on multiple shoot formation in pea plant with MS medium fortified with BAP and NAA as 1.0 and 0.2 mg/L respectively. These reported results strengthen our results and support the role of BAP in the shoot formation capacity of pea explants.

Callogenesis: Present study also included the callogeness response of pea explants. It is clear from the results after using different combinations of 2,4-D and KN, optimized callus induction and proliferation was noticed in MS medium supplemented with 2,4-D and lower conc. of KN. Cultures of Jannas and Climax cultivars of pea plant produces 60 and 70% of callus response respectively in MS medium supplemented with 2,4-D +KN as 5.0 and 0.5 mg/L respectively. Earlier different scientists have reported callogenic response of a number of explants of different plants with combination of 2,4-D and KN (Ramirez *et al.*, 2011; Dalila *et al.*, 2013; Hosseini *et al*; 2015). El Sayed (2011) described the formation of fine callus from pea plant using different explants. They reported the KN+2,4-D as good combination for callogenesis and provided 0.2:2 mg/L of KN and 2.4-D as the best combination or optimized one.

In another experiment for callus induction, a combination of 2, 4-D with BAP was also tried. In the present work, in cv. Janass, MS medium containing 2,4-D +BAP (3.0+0.5mg/L) gave 65% callus result from leaf explants while in case of cv. Climax 2,4-D +BAP (2.0+0.5mg/L) exhibited

70% callus induction response. There are earlier reports from Khan *et al. (*2013) about 71-97% callus response of chick pea explants. They used cotyledon as an explant. They reported 2, 4-D (4.0 mg/L) with BAP (5 µM) as an optimized medium. The effect of different plant growth regulators, their doses and combinations was also studied on callus response of *Tecomella undulata* (Sm) Seem (Patel and Patel, 2013). They used leaf as an explant and formulated a combination of BAP(3.0mg/L) and 2,4-D (0.5mg/L) as the best medium for callus formation with 91.2% response. Sundarasekar *et al.* (2012) reported 13.50 µM of 2, 4-D and 4.50 µM of BAP combination to be best for the initiation and production of friable callus (93.75%) in *Hymenocallis littoralis*. It can be concluded that a combination of auxin and cytokinin gave better results for callogenesis from leaf explants of two cultivars of pea.

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