Indian Journal of Ecology (2022) 49(5) (SI): 1925-1930
DOI: https://doi.org/10.55362/IJE/2022/3759

Manuscript Number: 3759
NAAS Rating: 5.79

Standardization of Micropropagation Protocol in Garden Pea (*Pisum sativum* L.)

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***Abstract*:** Garden Pea, being aleguminous vegetable crop is not very responsive to growth and regeneration under *in vitro* culture. Therefore, a reliable tissue culture protocol has always been difficult to establish in garden pea (*Vicia faba* L.) due to its recalcitrant nature. In the present investigation a rapid, reproducible and efficient tissue culture protocol was developed for garden pea. The variety used was P-89, a high yielding and widely cultivated garden pea variety developed by PAU, Ludhiana, Two explants i.e. seed and embryo were used for establishing aseptic cultures.The results obtained during different *in vitro* experiments revealed pre-treatment of seeds with Bavistin (0.2%) + Streptomycin (0.02%) for 10 minutes followed by 70% ethanol dip and 2% sodium hypochlorite treatment for 3 minutes resulted in highest survival for both the explants viz., embryo and seed(68.24 and 50.64%, respectively). Among all the regeneration medium, R_{16} - MS+2.00 mg/l BAP and 1.00 mg/l Kinetin was superior in terms of early shoot initiation and highest morphogenetic response in embryo (15.00 days and 73.33%) as well as seed (17.23 days and 53.76%). Embryo explant was more superior as compared to seed explant for culture establishment. In relation to multiple shoot formation, M_4 -MS + 2.00mg/l BAP + 1.00 mg/l TDZ produced the earliest and considerably superior response (19.00 days, 5.50 shoots/explant and.56 cm shoot length, respectively). For rooting, R_3 i.e. MS+ 1.00 mg/l IBA was found to be the optimum concentration forming maximum number of roots (3.67) and root length (4.24cm). The *in vitro* raised seedlings were hardened in plastic cups containing sterilized potting mixture comprised of FYM, sand and vermicompost in 1:1:1 ratio under partially controlled conditions in green house for 3 weeks before field transfer.

***Keywords*:** Feasibility**,** Garden Pea**,** *In vitro,* Sterilization, Tissue culture

Garden pea (*Pisum sativum* L.), a member of the Fabaceae family, is a widely cultivated cool-season vegetable crop in temperate and sub-tropical climates around the world. In the recent years, consumers and researchers have paid much attention to the nutritional and physiological benefits of peas and their by-products that are enhanced with biomolecules. It is preferable to develop pea cultivars with increased abiotic stress tolerance and pest and disease resistance. The Fabaceae species in general are difficult to regenerate *in vitro* due to recalcitrant nature, high genotypic specificity and are not amendable to *in vitro* propagation (Pratap et al 2018). The development of transgenic plants for many legumes is severely hampered by this recalcitrance towards in vitro regeneration since molecular genetics advances, such as gene over-expression, gene suppression, promoter analysis and T-DNA tagging, require effective transformation systems. Efficient tissue culture is an essential step in validating and using the data produced by these potent molecular tools. The pre-requisite for both genetic transformation and other tissue-culture derived techniques is the implementation of robust protocols for regeneration to generate genetic diversity such as somaclonal variation, *in vitro* mutagenesis, doubled haploids culture and wide hybridization (Dita et al2006). Very few or lesser attempts have been made to develop a reliable *in vitro* protocol in pea. The presence of totipotent tissues that react well to *in vitro* methods is a requirement for the success of tissue culture-based approaches in crop plants (Pratap et al 2010). Due to their juvenile nature, embryo culture is a convenient beginning for the establishment of shoot cultures and a critical milestone for mass micropropagation of plants from a small number of original seeds for many recalcitrant species (Khalafalla et al 2011). Establishment of aseptic cultures from field grown explants (seed, embryo, nodal cutting, leaf etc.) is first basic step for carrying out any further *in vitro* studies in the laboratory. Thus, the development of a specific sterilization procedure for field grown explants is a crucial step before identifying the growth stage for the collection of plant materials and perfecting the medium composition for multiplication of plants. Therefore, an attempt was made to develop a reliable and efficient protocol for obtaining rapid shoot multiplication using seed and embryo explants of Garden pea var. P-89 under *in vitro* conditions.

MATERIAL AND METHODS

Genotype and explants used: Two explants viz., seed and

embryo were used for standardization of micropropagation protocol in garden pea variety 'P-89'. The experiments were conducted in the Plant Tissue Culture Laboratory of the Division of Vegetable Science and Floriculture, SKUAST-Jammu during the year 2020-21.

Sterilization of explants: To standardize an effective sterilization technique for establishment of contamination free cultures in laboratory, the seeds were washed with water and few drops of Tween-20 for 15-20 minutes in order to clear the debris. The explants were surface sterilized with Bavistin (0.2%) + Streptomycin (0.02%) for 10 minutes followed by 70% ethanol dip and treatment with 2% NaOCl for varying time duration (1, 2 &3 minutes). Thereafter, explants were rinsed in distilled water for 4-5 minutes rapidly.

Different media used: Different concentration and combinations of growth regulators (auxins and cytokinin) have been used to optimize the media concentration for obtaining morphogenetic responses at various stages i.e. shoot initiation, shoot multiplication and elongation and finally rooting to obtain *in vitro* raised pea plantlets. The observations on different parameters were recorded after four weeks of culture.

Hardening: The *in vitro* raised seedlings were hardened in plastic cups containing sterilized potting mixture comprised of FYM, sand and vermicompost in 1:1:1 ratio underpartially controlled conditions in green house for 3 weeks before field transfer.

Culture conditions: All the cultures were incubated at 25°C with a 16 and 8-h light and dark photoperiod using cool white fluorescent tubes and an irradiance of 40 µmol m^{-2} s^{-1}.The photoperiod of incubation room was controlled using an electronic timer.

Statistical analysis: On the basis of observations averaged from 10 flasks/ test tubes per treatment replicated thrice, data was recorded from the *in vitro* experiments laid in Completely Randomized Design. Multiple comparison among the different treatments was undertaken following Tukey test using R studio software (R Core Team 2021).

RESULTS AND DISCUSSION

The results obtained from the various experiments conducted in Plant Tissue Culture laboratory to access the feasibility of *in vitro* seed / embryo culture for development of micropropagation protocol have been described in detail under the following sub-headings:

Refinement of sterilization procedure to enhance culture establishment: The fundamental step for conducting *in vitro* laboratory research is the establishment of aseptic cultures from field grown explants (seed, embryo, nodal cutting, leaf). It was essential to standardise the surface sterilization procedure before initiating tissue culture of *Pisum sativum*utilizing seeds and embryos as explants in order to establish aseptic cultures. It has been demonstrated that pre-treating explants with fungicides and bactericides before using surface sterilant significantly reduces contamination levels (Panathula et al 2014). Ethanol (70%) and sodium hypochlorite (2%) were employed in combination as two surface sterilant for varying periods of time (1, 2 and 3 minutes). The different sterilizing treatments affect the percentage of seeds and embryo explants that survive (Table 1). The treatment combination with the highest survival rate for both explants, the embryo (68.24%) and seed (50.64%), consisted of pre-treatment with Bavistin (0.2%) and Streptomycin (0.02%) for 10 minutes, followed by an ethanol dip (70%) and sodium hypochlorite (2%) treatment for 3 minutes. The results further revealed that when exposed to any change in time period, explants' survivability significantly decreased. The treatment combination comprising of 5 minutes pre-treatment with Bavistin (0.2%) and streptomycin (0.02%) followed by 70% ethanol dip and sodium hypochlorite (2%) treatment for 1 minute recorded minimum survival percentage in both the explants viz., embryo (5.01%) and seed (3.28%). The findings are in accordance with the research of Firoz et al (2016) and Zinabu et al (2018) on the sterilization of cucumber seeds and inset explants, respectively.

Effect of different explants and MS media concentrations on shoot initiation: For shoot induction using embryo and seed as explants, 18 different MS medium concentrations fortified with various dosages of BAP alone (0.50, 1.00, 1.50, 2.00, 3.00, and 4.00 mg/l) and in conjunction with Kinetin (0.50 and 1.00 mg/l) were used. The

Table 1. Refinement of sterilization procedure to enhance culture establishment

Time duration		Explant survival (%)	
Pre-treatment with 0.2% Bavistin + 0.02% Streptomycin	70% ethanol dip followed by treatment with 2 % NaOCl	Embryo	Seed
5	1 minute	5.01^{h}	3.28^{h}
	2 minutes	10.00^{g}	6.05^{g}
	3 minutes	20.76^{e}	14.00^{f}
10	1minute	34.56^{d}	25.75^{d}
	2 minutes	56.69^{b}	41.16^{b}
	3 minutes	68.24^{a}	50.64^{a}
20	1minute	55.70^{b}	25.48^{d}
	2 minutes	47.50^{c}	28.58^{c}
	3 minutes	15.04^{f}	16.04^{e}

Means of all the characters followed by different letters within a column are significantly different accordingly to Tukey's test ($P < 0.05$)

morphogenetic response varied greatly, ranging between 12.67 to 73.33% in case of embryo explants and 8.67 to 53.76% in seeds. Among all the regeneration medium, significantly higher morphogenetic response for both the explants was recorded in R_{16}- MS medium fortified with 2.00 mg/l BAP and 1.00 mg/l Kinetin (73.33, 53.76) followed by two regeneration medium, viz., R_{10} – MS medium fortified with 2.00 mg/l BAP and 0.50 mg/l Kinetin (70.67, 51.00) and R_{11}- MS medium fortified with 3.00 mg/l BAP and 0.50 mg/l Kinetin (64.00,50.33). The lowest morphogenetic response was observed in regeneration medium, R_1, which is MS medium enriched with 0.50 mg/l BAP (12.67 and 8.67, respectively.)As regards to earliness of the response, expressed as days taken to shoot initiation, minimum number of days to shoot initiation (15.00 and 17.23) was recorded in R_{16}-MS medium fortified with 2.00 mg/l BAP and 1.00 mg/l Kinetin which outperformed all other medium. However, maximum days taken to shoot initiation (26.25 and 28.52) was recorded in R_1 - MS basal medium fortified with 0.50 mg/l BAP among all the medium. The overall best and earliest shoot initiation response was obtained in regeneration medium R_{16} i.e., MS medium fortified with 2.00 mg/l BAP + 1.00 mg/l Kinetin in embryo explants (Table 2, Plate1, A and B) and with profuse shoot primordia emergence when seeds were used as explants (Plate 1: C and D).The results revealed that for *in vitro* shoot initiation, embryo explants were superior to seeds, with a higher morphogenetic response and earlier shoot induction. The study corroborate with the findings of Rajput and Singh (2010) that pea embryo explants were superior than other explants.

Effect of various MS media concentrations and combinations on shoot multiplication: Single micro shoots (3-4 cm) were inoculated on MS media fortified with BAP (2.00, 3.00, and 4.00 mg/l) and TDZ (0.50 and 1.00 mg/l) (Table 3). Minimum days to multiple shoot formation (19.00) were in M_4 - MS medium fortified with 2.00 mg/l BAP and 1.00 mg/l TDZ followed by M_1-MS medium fortified with 2.00 mg/l BAP and 0.50 mg/l TDZ (22.17). The maximum number of days to multiple shoot development (28.50) were observed in the multiplication medium, M_6- MS media enriched with 4.00 mg/l BAP and 1.00 mg/l TDZ. However, in multiplication medium, M_6- MS medium enriched with 4.00 mg/l BAP and 1.00 mg/l TDZ, the maximum days to multiple shoot development (28.50) were observed. The maximum number of shoots per explant were in M_4 - MS medium fortified with 2.00 mg/l BAP and 1.00 mg/l TDZ, followed by M_5 - MS medium fortified with 3.00 mg/l BAP and 1.00 mg/l TDZ (4.50), while rest of the medium exhibited an average

Table 2. Effect of different explants and MS media concentrations on shoot initiation

Regeneration medium R*	Concentrations used (mg/l)		Morphogenetic response (%)		Days taken to shoot initiation	
	BAP	Kinetin	Embryo	Seed	Embryo	Seed
R_1	0.50	0.00	12.67^{k}	8.67^{a}	26.25^{j}	28.52^{a}
R_2	1.00	0.00	18.33^{j}	16.67^{b}	25.25^{i}	27.67^{ab}
R_3	1.50	0.00	20.00^{j}	28.33^{bc}	24.00^{h}	26.00^{bc}
R_4	2.00	0.00	38.67^{i}	33.00^{bc}	24.45^{g}	28.00^{ab}
R_5	3.00	0.00	40.33^{hi}	35.67^{cd}	24.65^{fg}	21.00^{efg}
R_6	4.00	0.00	49.67^{g}	30.00^{cd}	23.56^{h}	20.54^{fg}
R_7	0.50	0.50	50.00^{def}	38.33^{cd}	23.66^{ef}	22.54^{ef}
R_8	1.00	0.50	54.00^{ef}	39.67^{cd}	22.00^{e}	20.43^{fg}
R_9	1.50	0.50	56.33^{def}	43.67^{cde}	22.66^{d}	20.00^{g}
R_{10}	2.00	0.50	70.67^{a}	51.00^{i}	17.00^{ab}	22.00^{efg}
R_{11}	3.00	0.50	64.00^{b}	50.33^{hi}	18.00^{b}	22.34^{ef}
R_{12}	4.00	0.50	61.00^{bc}	46.33^{def}	21.67^{cd}	25.00^{cd}
R_{13}	0.50	1.00	52.33^{fg}	39.00^{def}	22.18^{e}	20.21^{fg}
R_{14}	1.00	1.00	57.33^{cde}	44.00^{ef}	21.00^{d}	21.95^{efg}
R_{15}	1.50	1.00	58.67^{cd}	45.33^{fg}	20.00^{d}	23.00^{de}
R_{16}	2.00	1.00	73.33^{a}	53.76^{j}	15.00^{a}	17.23^{h}
R_{17}	3.00	1.00	43.33^{h}	48.67^{gh}	19.00^{bc}	20.45^{fg}
R_{18}	4.00	1.00	17.33^{j}	46.00^{bc}	24.00^{cd}	21.24

Means of all the characters followed by different letters within a column are significantly different accordingly to Tukey's test ($P < 0.05$)

response. However, M_3-MS media fortified with 4.00 mg/l BAP and 0.5 mg/l TDZ showed the minimum number of shoots per explant (3.01). Multiplication medium, M_4- MS medium supplemented with 2.00 mg/lBAP and 1.00 mg/l TDZ recorded the maximum average length of shoot (3.56 cm), followed by M_5- MS medium enriched with 3.00 mg/l BAP and 1.00 mg/lTDZ (2.92 cm) whereas minimum length of shoot (1.96 cm) was recorded in M_3- MS medium fortified with 4.00 mg/l BAP and 0.50 mg/l TDZ. Therefore it was revealed that, among all the multiplication medium, medium M_4 with MS media fortified with 2.00 mg/l BAP and 1.00 mg/l TDZ provided the earliest and greatest outcomes in terms of multiple shoot development (Plate 2). However, at higher BAP concentrations, a noticeable drop in the number of shoots and the average length of shoots was observed. This reduction might be attributed to larger doses of BAP not expressing superiority because of their detrimental effects at higher concentrations (Waseem et al2011). The synergistic effect of BAP and TDZ on the development of multiple shoots has been observed in chrysanthemum by Sushmarani et al (2021).

Effect of different concentrations of IBA on rooting response: Individual shoots were cultured on MS medium enriched with different doses of IBA (0.50, 1.00, 1.50, and 2.00 mg/l) to induce roots in *in vitro* multiplied shoots and the data regarding differential response of various rooting media has been shown in Table 4. The rooting media R_2- MS

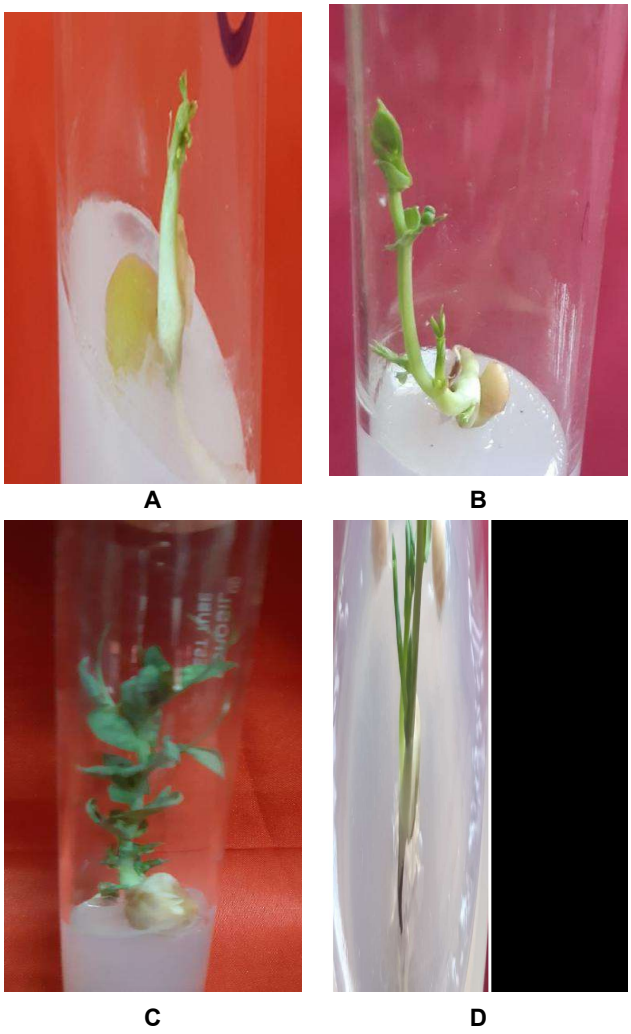

Plate 1. A, B,C and D: Shoot initiation from different explants (embryo & seed) in MS medium fortified with 2.00 mg/l BAP + 1.00 mg/l Kinetin

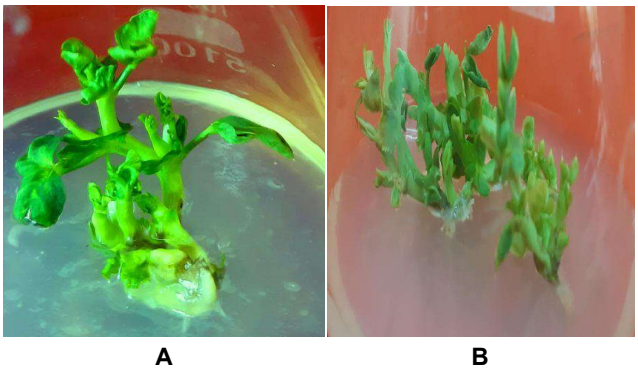

Plate 2. A and B: Multiple shoot formation on MS medium fortified with 2.00 mg/l BAP + 1.00 mg/l TDZ

Table 3. Effect of various MS media concentrations and combinations on shoot multiplication

Multiplication medium M*	Concentrations used (mg/l)		Days taken to formation of multiple shoot	No. of shoots/ explant	Average length of shoot (cm)
	BAP	TDA			
M_1	2.00	0.50	22.17^{d}	4.11^{c}	2.77^{bc}
M_2	3.00	0.50	24.00^{c}	4.00^{c}	2.56^{c}
M_3	4.00	0.50	25.50^{b}	3.01^{e}	1.96^{e}
M_4	2.00	1.00	19.00^{e}	5.50^{a}	3.56^{a}
M_5	3.00	1.00	$25.00b^{c}$	4.50^{b}	2.92^{b}
M_6	4.00	1.00	28.50^{a}	3.50^{d}	2.22^{d}

Means of all the characters followed by different letters within a column are significantly different accordingly to Tukey's test ($P < 0.05$)

Table 4. Effect of different concentrations of IBA on rooting response

Rooting medium R*	Concentrations used IBA (mg/l)	No. of roots / shoot	Average length of root (cm)
R_0	0.00	1.04[d]	2.02[d]
R_1	0.50	2.50[b]	3.11[b]
R_2	1.00	3.67[a]	4.24[a]
R_3	1.50	1.96[c]	2.50[c]
R_4	2.00	1.23[d]	2.02[d]

Means of all the characters followed by different letters within a column are significantly different accordingly to Tukey's test ($P < 0.05$)

A B

Plate 3. A and B: Root development on MS medium + 1.00 mg/l IBA

medium fortified with 1.00 mg/l IBA worked better than any other medium, generating the maximum number of roots/shoots (3.67), and was followed by R_1- MS medium fortified with 0.50 mg/l IBA (2.50) while rest of the rooting medium showed average to below average rooting response, R_0-MS basal medium exhibited poor response and produced only 1.04 roots per shoot. R_2- MS medium enriched with 1.00 mg/l IBA also had the highest root length (4.24 cm), followed by R_1- MS medium fortified with 0.50 mg/l IBA (3.11cm). Thus, it can be inferred that, of all the medium tested, R_2-MS medium enriched with 1.00 mg/l IBA showed the best rooting response (Plate 3). The rooting response decreased with rise in level of IBA concentrations may be due to the fact that roots need lower concentration of auxin for growth, but more auxin slows root growth significantly since it causes the generation of ethylene, which inhibits root growth at this concentration (Taiz and Zeiger, 2002). The results in harmony with those of Khalafalla et al (2010) and Mohapatra et al (2018).

CONCLUSION

A reliable and efficient *in vitro* protocol has been standardized for stages such as shoot bud initiation, multiplication, and rooting by using different hormonal concentrations to obtain micro propagated pea plantlets. It can be concluded that sterilization procedure consisting of pre-treatment of seeds with Bavistin (0.2%) + Streptomycin (0.02%) for 10 minutes followed by 70% ethanol dip and 2% sodium hypochlorite treatment for 3 minutes resulted in highest survival for both the explants viz., embryo and seed. Best shoot initiation response was observed on MS media fortified with 2.00 mg/l BAP and 1.00 mg/l Kinetin in both the explants. Embryo explant was found to be more superior as compared to seed explant for culture establishment. On MS medium fortified with 2.00mg/l BAP and 1.00 mg/l TDZ, minimum days to formation of multiple shoots (19.00), highest no. of shoots/ explant (5.50) and average length of shoot (3.50cm) were reported for shoot multiplication. For rooting, MS medium fortified with 1.00 mg/l IBA was found to be the optimum concentration forming maximum number of roots (3.67) and root length (4.24cm). Finally, it can be stated that the present investigation will be a boon to pea growers in the Jammu region, as it will provide quality planting material which could be further used in the crop improvement programmes and genetic transformation studies.

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Received 19 August, 2022; Accepted 15 September, 2022

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