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Optimization of *Brassica oleracea* var. Pusa Snowball Using Seedling Explants for the *in vitro* Regeneration Protocol

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ABSTRACT

Cauliflower is an important Cole crop grown in India but the attack of insects-pests hampers the production of the crop. Generation of the transgenic crop resistant to insects can serve to be a solution for the problem. Therefore, establishment of an optimized *in vitro* regeneration protocol of the Indian cauliflower is a pre-requisite, hence, two explants, namely, cotyledon and hypocotyls were obtained from the seedlings of the Indian cauliflower variety Pusa Snowball K-1. Results showed that the shoot regeneration from hypocotyls explants on media containing 1 mg/l BAP along with the combination 0.1 mg/l IAA was best suited and for root regeneration 1.5 mg/l IBA was found to be most effective. Further, standardized protocol complemented for *Agrobacterium*-mediated transformation against the insect Diamondback moth i.e. the DBM.

Key words: *In vitro* regeneration, Indian cauliflower, hypocotyl, cotyledon, diamond back moth

INTRODUCTION

The micro propagation is a highly reproductive technique and its aim lies not only in producing virus-free plants but also aids in developmental studies for various other tissue and organ cultures, agricultural development in genetic engineering and so on. *Brassica oleracea* is among the species belonging to the vast and diversified family of Brassicaceae (Kumar and Srivastava, 2016). This includes crops like, cauliflower, cabbage, turnip, kohlrabi, broccoli, etc, which are consumed in a relatively good quantity by a large population worldwide (Mabry *et al.*, 2021). Due to their nutritional value, they serve to be a good source of calcium and vitamin-C, moreover, are low in fats and also considered to have important proteins and vital vitamins and minerals in addition to their fiber content which has gained popularity in a larger section (Sanlier and Guler, 2018). The traditional hybridization breeding methods involve a tedious process, moreover, it is time consuming, expensive and the two-year long head-seed complicated cycle as well (Shaw *et al.*, 2021). Hence, the conventional approach of breeding practices needs improvements and additions such that they are replaced by the new and improved biotechnological and genetically advanced methods and techniques. One of the routine procedures to protect the crop from insect-pests is spraying of large quantities of insecticides (Shende, 2016). But, the concern arises that usage of huge quantities of such harmful insecticides and pesticides does not suffice the purpose and also poses a health threat to humans and in addition is not good for our environment and ecosystem (Sharma *et al.*, 2019). Thus, there is an urgent call for finding an alternative to control the severe damages being caused by the pests (Sharma *et al.*, 2020).

In this study, the plant regeneration protocol for the Indian cauliflower was standardized which produced shoots and roots, and further, produced disease-free plants. The shoots were regenerated by the explants cotyledon and hypocotyls excised from the 7-8 days seedlings that germinated on the MS media. However, even while working on the same variety different groups have encountered varied changes and thus, the regeneration protocols need to be standardized according to the specific genotype (Gerszberg *et al.,* 2015). It is noteworthy that for an efficient *in vitro* culture of most *Brassica* species the type of genotype used and the various growth regulators greatly influence the success of regeneration (Mandingbam *et al.*, 2020). The shoot regeneration has also been reported from leaf and root segments (Rahman *et al.,* 2021), hypocotyls and cotyledons as well (Gambhir *et*

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al., 2017; Rahman *et al.,* 2021). The maintenance of the cauliflower parent line has also been done by the micro propagation using curd meristem as an explants (Sheng *et al.,* 2022), petals, leaf and various tissues in Brassica (Kumar and Srivastava, 2015). Different types of explants have been used as a starting material for the cauliflower micro propagation such as, seedling explants (Gambhir *et al.*, 2017), anther culture (ZhiPing *et al.*, 2016), protoplast culture (*Gerszberg,* 2018), roots (Gerszberg *et al.,* 2015; Rahman e*t al.,* 2021) and peduncles (*Gerszberg,* 2018).

The regeneration protocol was optimized in *Brassica oleracea,* var. *botrytis*, in the specific genotype, Pusa snowball K-1 (PSBK-1) to raise and maintain a disease-free stock of plants that were later used for genetic transformation experiments required to develop resistance against the biotic stress, specific to insect resistance against the *Plutella xylostella*. The different hormonal combinations along with the varied concentrations were studied and observed using MS culture media.

MATERIALS AND METHODS

Standardization of the conditions for the *in vitro* regeneration of the Indian cauliflower was done and for this purpose Indian cauliflower, namely, PSBK-1 was used. The seeds were obtained from Katrain, Himachal Pradesh, India. The seeds were rinsed in tap water followed by disinfecting the seeds with 70% Ethanol v/v for 1 min. Further washed in 3-4 drops of Cween 20 detergent for 2-3 min and then, thoroughly rinsed with autoclaved double distilled water for 3-4 times to wash off the detergent completely. 0.1% v/v Sodium Hypochlorite was used to rinse the seeds for 10 min and washed properly 5-7 times with autoclaved double distilled water to shed off the sodium hypochlorite solution. The seeds were then placed on autoclaved Wattmann filter paper for drying inside the laminar airflow cabinet.

The seeds were then used for germination in the autoclaved jam bottles containing the MS (Murashige and Skoog, 1962) basal germination media and sealed with parafilm. The basal medium for seeds was kept plant growth regulator free and the germination medium contained a mixture of inorganic and organic salts involving nutrients from the MS media, containing 20 g/l sucrose as the carbon source and 7 g/l agar was used as a solidifying agent. The pH of the medium was maintained to 5.7-5.8 and then autoclaved before being used. The seeds germinated at 23°C using a 16-h photoperiod with a light intensity of 50 mm E/m^2/s supplemented by white fluorescent lamps. The explants cotyledon and hypocotyl were aseptically excised from the 7-8 day germinated seedlings. The excision of 2-3 mm long cotyledon and 7-8 mm length of hypocotyl explants was done via autoclaved forceps and scalpel. Each explant was embedded in the culture medium and approximately 20 explants were inoculated in a single 90 mm pteriplate. Different combinations and concentrations of varied hormones were used to find out the effect and best-suited combination for callus, shoot and root regeneration. The various hormonal combinations are given in Tables 1, 2 and 3. The experimentation was conducted at 22-23°C under a 16-h photoperiod regime of cool white fluorescent light (50 mm E/m^2/s). The cultured cotyledon and hypocotyl explants were sub-cultured on a regular interval for growth. All the phytohormones used in varied combinations were prior filter-sterilized (Millipore 0.22 mm) and added to the autoclaved basal media. The basal media contained 2% (w/v) sucrose, solidified with 0.8% (w/v) agar and pH adjusted to 5.7-5.8 was autoclaved (121°C, 15 psi, 20 min). As callogenesis was observed initially the cultures were kept in dark to avoid the browning of the tissues due to the release of phenolic components. When the whole tissue proliferated then a 16-h photoperiod regime was strictly followed.

Table 1. List of the media compositions for *in vitro* callus induction

S. No.	Media code	Media composition
1.	CIM 1	MS+2,4-D (1 mg/l)
2.	CIM 2	MS+2,4-D (1.5 mg/l)
3.	CIM 3	MS+2,4-D (2 mg/l)
4.	CIM 4	MS+2,4-D (2.5 mg/l)
5.	CIM 5	MS+BAP (0.5 mg/l)
6.	CIM 6	MS+BAP (1 mg/l)
7.	CIM 7	MS+BAP (1.5 mg/l)
8.	CIM 8	MS+NAA (0.5 mg/l)
9.	CIM 9	MS+NAA (1 mg/l)
10.	CIM 10	MS+KIN (1 mg/l)
11.	CIM 11	MS+KIN (1.5 mg/l)
12.	CIM 12	MS+KIN (2.0 mg/l)
13.	CIM 13	MS+2,4-D (1 mg/l)+BAP (0.5 mg/l)
14.	CIM 14	MS+2,4-D (1 mg/l)+KIN (0.5 mg/l)
15.	CIM 15	MS+2,4-D (1.5 mg/l)+KIN (0.5 mg/l)
16.	CIM 16	MS+2,4-D (1 mg/l)+NAA (0.5 mg/l)
17.	CIM 17	MS+BAP (1 mg/l)+NAA (0.5 mg/l)+KIN (1 mg/l)+IAA (0.5 mg/l)

Table 2. List of media combinations for shoot induction

S. No.	Media code	Media composition
1.	SIM 1	MS+BAP (0.5 mg/l)
2.	SIM 2	MS+BAP (1 mg/l)
3.	SIM 3	MS+BAP (1 mg/l)+NAA (0.1 mg/l)
4.	SIM 4	MS+BAP (1 mg/l)+IAA (0.1 mg/l)
5.	SIM 5	MS+BAP (2 mg/l)
6.	SIM 6	MS+BAP (2 mg/l)+NAA (0.1 mg/l)
7.	SIM 7	MS+BAP (2 mg/l)+IAA (0.1 mg/l)

Table 3. List of the media combinations for rooting

S. No.	Media code	Media composition
1.	RI 1	MS
2.	RI 2	MS+IBA (0.5 mg/l)
3.	RI 3	MS+IBA (1 mg/l)
4.	RI 4	MS+IBA (1.5 mg/l)

With the occurrence of fresh callus (Table 1), these were transferred to the various shoot regeneration media compositions (Table 2). The table shows the various combinations and concentration of the growth regulators for shoot induction. The elongated shoots were further separated and cultured into the rooting media containing varied concentrations of Indole-3-Butyric Acid (IBA; Table 3). The plantlets which attained a size of 7-8 cm were then potted and placed in a small pot covered with plastic sheets in order to maintain high humidity. After 10-12 days, the plants were shifted to the greenhouse for further growth and development.

RESULTS AND DISCUSSION

The seeds of *B. oleracea* successfully germinated on the plant growth regulator free MS medium and the percentage of germination was observed to be high (97%) after 7-8 days of germination. On the callus-induction media, the explants became swollen and expanded in size later, they were placed on a callus multiplication medium. The best treatment combinations for the regeneration protocol from callus to the rooting stage are given below in Table 4. The callus when increased in size gave rise to the shoots in the shoot regeneration medium. MS+BAP (1 mg/l)+IAA (0.1 mg/l) induced the highest percentage of plants producing shoots (95%) in hypocotyl (Table 5). Regular sub-cultures were done at an interval of 12-14 days which led to increase in the number of regenerated shoots. In general, the hypocotyl showed better results than cotyledon with respect to the shoot formation as 95% regeneration frequency was observed, whereas in cotyledon 73% was observed (Table 5). The presence of BAP showed an increase in the number of shoots produced per explant in *B. oleracea in vitro*. For root induction, the shoots that attained a size of approximately 4-5 cm in height were transferred onto a plant growth regulator free medium as well as in the medium containing three different concentrations of IBA. Root formation occurred within 12-20 days after transferring shoots to the rooting media. It was observed that treatment containing MS+IBA (1.5 mg/l) gave the highest percentage of explant- producing roots (Table 6).

The rooted plantlets were further acclimatized in the potting mixture. The plants were

Table 4. List of the best treatment combinations for the regeneration protocol

S. No.	Treatment code	Regeneration stage	Treatment combination and concentration
1.	CIM 1	Callus induction	MS+2,4-D (1 mg/l)
2.	CMM 5	Callus multiplication	MS+BAP (5 mg/l)+NAA (0.5 mg/l)
3.	SIM 5	Shoot regeneration	MS+BAP (1 mg/l)+IAA (0.1 mg/l)
4.	RI 4	Rooting	MS+IBA (1.5 mg/l)

Table 5. Production of shoots on various hormonal combinations from cotyledon and hypocotyl of *B. oleracea*

S. No.	Treatment	Cotyledon		Hypocotyl	
		% of responsive explants	No. of shoots/ explant	% of responsive explants	No. of shoots/ explant
1.	MS+BAP (0.5 mg/l)	48	2±0.15	52	4±0.42
2.	MS+BAP (1.0 mg/l)	52	3±0.24	62	5±0.62
3.	MS+BAP (1.0 mg/l)+NAA (0.1 mg/l)	44	2±0.18	26	1±0.30
4.	MS+BAP (1.0 mg/l)+IAA (0.1 mg/l)	73	12±1.15	95	15±1.25
5.	MS+BAP (2.0 mg/l)	62	5±0.68	70	8±0.82
6.	MS+BAP (2.0 mg/l)+NAA (0.1 mg/l)	50	3±0.52	65	7±0.80
7.	MS+BAP (2.0 mg/l)+IAA (0.1 mg/l)	30	1±0.14	32	3±0.20

Table 6. Production of roots on various hormonal combinations from cotyledon and hypocotyl of *B. oleracea*

S. No.	Treatment	Cotyledon		Hypocotyl	
		% of shoots rooted	Avg. no. of roots	% of shoots rooted	Avg. no. of roots
1.	MS	45	2±0.12	63	4±0.48
2.	MS+IBA (0.5 mg/l)	35	1±0.24	53	3±0.42
3.	MS+IBA (1 mg/l)	61	3±0.32	70	5±0.68
4.	MS+IBA (1.5 mg/l)	77	4±0.28	96	8±1.12

excised from the jar bottles gently and washed with tap water to remove the agar particles. The plants having root systems were put in the test tubes containing autoclaved distilled water for 2-3 days for acclimatization. A mixture of sand, vermiculite, coco peat and perlite (6 : 3 : 2 : 1) was made as a potting mixture and this mixture along with the pots was autoclaved at 121°C for 20 min at 15 psi. The potting mixture was filled in the pots once the mixture cooled down and a deep hole was made in the soil to transplant the grown plants. The regenerated plants were gently put in the soil with roots deep dug in the potting mixture and each pot was labelled properly. These pots were safely transferred to the National Phytotron Facility, New Delhi, where they were maintained at 23°C (Fig. 1, pictorial presentation of the protocol).

The results showed a successful *in vitro* regeneration protocol thus, so developed in the Indian cauliflower from the seedlings explants used. Therefore, it is evident that the hormones, their combinations and varied concentrations play an important and indispensable role in the regeneration of shoots and roots and later, obtaining the establishment of a plant. As observed the effectiveness of these hormones was considerably variable in the two different explants, hence, one can conclude that the responsiveness of the hormones and their interaction, greatly affects the explants growth, induction of shoots and roots and their responsive percentage as well.

In conclusion, the results showed satisfactory performance in producing a good frequency of shoot regeneration from hypocotyl explants and multiplication of shoots on media containing 1

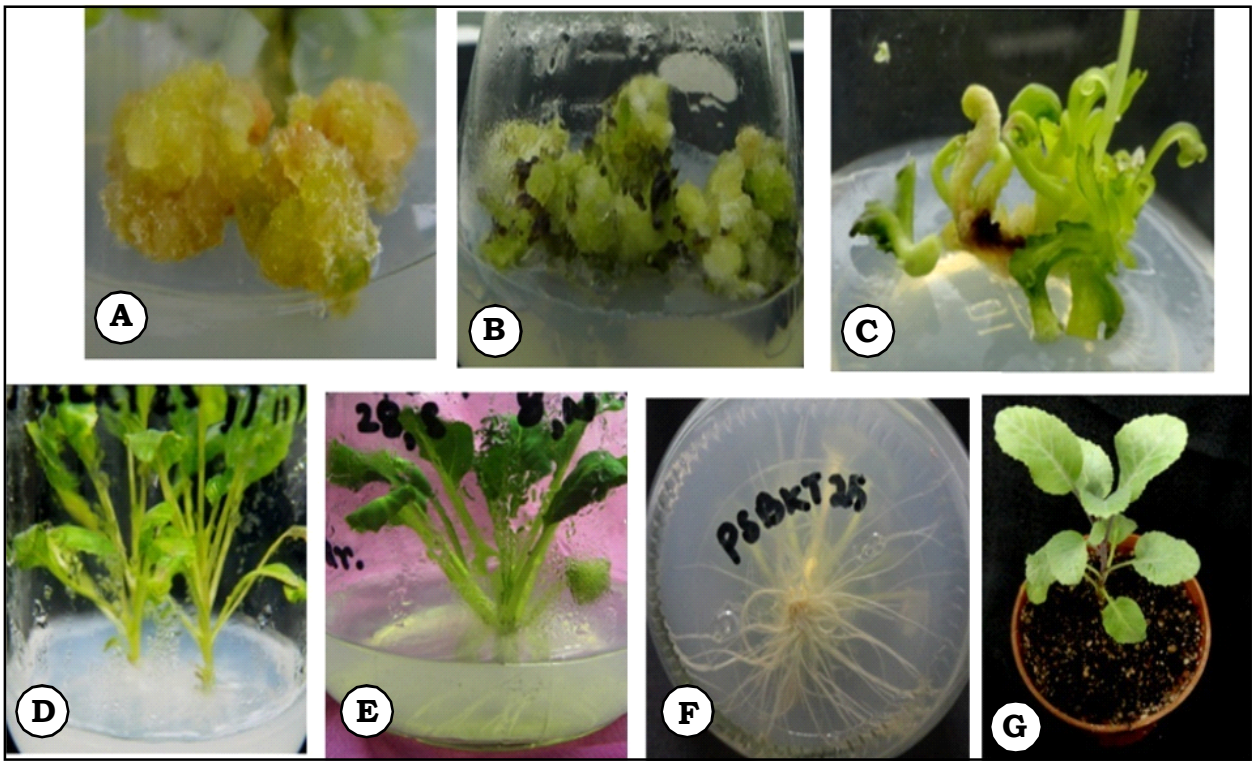

Fig. 1. Stages of *in vitro* regeneration of Indian cauliflower (A) : Callus induction, (B) and (C) : Shoot initiation, (D) : Shooting, (E) and (F) : Rooting and (G) : Hardening and Acclimatization.

mg/l BAP along with the combination 0.1 mg/l IAA. Root regeneration was best obtained in 1.5 mg/l IBA. IBA is reported to be the superior and the most effective auxin for root initiation in comparison to IAA or NAA (Gambhir *et al.*, 2017). This efficient *in vitro* plant regeneration, protocol established was further useful in developing transgenic plant lines against the insect resistance in a shorter period of time frame. The explant was used for transformation via co-cultivation with the *Agrobacterium tumefaciens* containing the desired construct. This protocol aided in genetic improvement by using biotechnological approaches and thus, this protocol was applied this protocol for generating *B. oleracea*. Pusa Snowball variety with improved tolerance to biotic stress, specific to the insect *Plutella xylostella* also popularly known as Diamondback moth. Therefore, with the help of this regeneration protocol, high percentages of transgenic plants were rapidly obtained. In addition, the protocol will not only aid in the study of *in vitro* plant regeneration through tissue culture technique but would also act as a tool for the researchers for greatly enhancing the production of healthy, uniform and disease free plants.

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