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In vitro propagation of a self-incompatible cabbage line 'Sel. 5'

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ABSTRACT

A viable protocol was developed for *in vitro* maintenance and multiplication of cabbage SI line 'Selection-5' with very strong S-allele interaction. Different types of explants, *viz*., apical bud, axillary bud and basal shoot sprout were tested for their *in vitro* regeneration ability. The morphogenetic potential varied among the explants. Apical bud proved to be the most potent explant for initial culture establishment followed by axillary bud. Murashige and Skoog (1962) medium supplemented with 2 mg l^{-1} BA, 0.5 mg l^{-1} NAA and 0.1 mg l^{-1} GA_3 was optimum for culture establishment. The maximum *in vitro* shoot proliferation (5.89 ± 0.38) was obtained on MS medium supplemented with 5 mg l^{-1} KIN + 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} GA_3. The proliferation rate was significantly influenced by type and concentration of cytokinins with kinetin being more effective than BA. Half-strength MS medium supplemented with 1.0 mg l^{-1} IBA was most effective for rooting. The tissue cultured plants were successfully hardened and transferred to field conditions with a survival rate of 76.67 per cent.

Key words: Cabbage, self-incompatibility, micropropagation, growth hormones.

INTRODUCTION

Cabbage (*Brassica oleracea* var. *capitata* L.) is a major winter season crop in the Indian sub-continent and presently in cultivation through out the year after availability of high temperature tolerant F_1 hybrids. Two pollination control mechanisms namely, self-incompatibility (SI) and cytoplasmic male sterility (CMS) are presently in use for the production of hybrid seeds. Until now, all cabbage hybrid seeds have been produced using self-incompatible lines all over the world (Fang *et al.*, 4). However, maintenance of SI lines is very tedious. Bud pollination, CO_2 treatment or NaCl spray is required to overcome incompatibility and maintain SI lines in homozygous form (Kucera, 5).

The *in vitro* tissue culture offers a powerful aid for the acceleration of cabbage plant production, especially when referring to parental lines of F_1 hybrids that have strong SI background (Cristea *et al*., 3). It eliminates the need for the elaborate and expensive procedure for establishing and maintaining sib-incompatible nearly homozygous parental breeding lines. Various factors such as explant, hormone combination and concentration and cultural conditions affect the rapid *in vitro* propagation of self-incompatible line in headed cabbage (Zhang *et al.*, 13). Shoot regeneration has been achieved from various tissues and organs including hypocotyl, cotyledon, root, leaf, peduncle segment, callus and cell culture, thin cell layers and protoplasts (Cardoza and Stewart, 1). However, considerable variation has been observed by different groups, even when working with the same species or variety. With the aim to improve the breeding process of cabbage and to develop new cultivars in a shorter period of time, as well as to develop a basis for use of biotechnological methods, the *in vitro* propagation of cabbage from different explants, was investigated.

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MATERIALS AND METHODS

The present study was conducted on SI cabbage line 'Sel-5', which is a good combiner and has very strong SI system. The mother plant was raised under open field condition at the Research Farm, IARI, Regional Station Katrain, Kullu, Himachal Pradesh. Three different types of explants, *viz*., shoot apex or apical bud, axillary bud and basal shoot sprout were used for initial culture establishment. The apical bud, axillary bud and basal shoot sprout were collected after bolting from selected mother plants during the year 2009. Well prepared explants were washed with Teepol® (0.1%) solution for 5 min. followed by thorough washing under running tap water for 10-15 min. to remove any residue of the detergent. The explants were pre-treated with 0.1% carbendazim (Bavistin®), 100 mg l^{-1} cetrimide and 200 mg l^{-1} 8-hydroxy quinnoline citrate for two hours. They were surface sterilized with 0.1 percent mercuric chloride for 10 min. followed by 4-6 washings with sterile double-distilled water to remove the traces of sterilizing agents after treatment.

Murashige and Skoog (MS) medium (Murashige and Skoog, 7) containing 30 g sucrose, solidified

with 8 g l^{-1} agar and pH adjusted to 5.75 (before autoclaving) was used at full and half strengths for different experiments. All the media were sterilized by autoclaving at 121ºC for 20 min. (15 psi). The explants were initially inoculated on MS medium supplemented with 2 mg l^{-1} 6-benzyl adenine (BA), 0.5 mg l^{-1} α-naphthalene acetic acid (NAA) and 0.1 mg l^{-1} gibberellic acid (GA_3). The cultures were maintained at 25 ± 1ºC under fluorescent white light (47 µmol $m^{-2}s^{-1}$) at a photoperiod of 16:8 h light and dark cycles. Twenty explants were inoculated per replication and each experiment was replicated thrice.

After four weeks of initial establishment, the sprouted shoots were separated and multiplied on MS medium supplemented with different concentration of BA (1, 3 and 5 mg l^{-1}) or kinetin (KIN: 1, 2, 3, 4 and 5 mg l^{-1}) along with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} GA_3. The shoot proliferation and length was recorded after four weeks of transfer on to the shoot proliferation media. The multiplied micro-shoots were dissected and transferred on to shoot elongation media comprising of basal MS medium supplemented with GA_3 (0.25, 0.5, 1.0 and 2.0 mg l^{-1}).

In order to induce roots, the proliferated shoots containing 3-5 leaves were dissected and inoculated in half-strength MS medium supplemented with indole-3-butyric acid (IBA) and NAA at concentration of 0.25, 0.5, 1.0 or 2 mg l^{-1} (either singly or in combination). Fifteen-day-old rooted plantlets were carefully removed from flasks. Roots were rinsed with running water to eliminate residue from the culture medium and then soaked in a fungicidal solution (carbendazim 0.1%) for five minutes. Subsequently, plantlets were transferred to the hardening media consisting of sterilized peat and Soilrite mixture (1:1) saturated with half-strength MS salts (macro and micro) only. The survival rate of the acclimatized plants was recorded three weeks after transplanting.

The data were subjected to standard analysis of variance (ANOVA) to test significance of different traits across different concentrations and combinations of growth regulators. Duncan's multiple range test was also employed to find significant differences among means of traits.

RESULTS AND DISCUSSION

The different explants were compared for their regeneration ability on MS medium supplemented with 2 mg l^{-1} BA, 0.5 mg l^{-1} α-NAA and 0.1 mg l^{-1} GA_3 (Table 1). The morphogenetic potential varied among the explants. Apical bud (Fig. 1a) proved to be the most potent explant for initial culture establishment followed by axillary bud. The maximum culture establishment (83.33%) was recorded in apical and axillary bud. Basal shoot sprout showed poor culture establishment. The reason for poor establishment in basal shoot sprout may be the high rates of microbial contamination. Whereas, apical and axillary buds were more shielded inside the leaves and petiole and hence showed less microbial contamination. Shoot sprouting or development of adventitious bud in apical and axillary bud was observed after 9.07 and 10.33 days, respectively.

The highest number (7.20 ± 1.60) of shoots regenerated per explant was obtained in apical bud. The regeneration potential of basal shoot sprout was quite poor (3.73 ± 1.50). Apical and axillary bud explants were at par for their ability to regenerate and produce multiple shoots. Both these explants differed significantly from basal sprout. Cabbage plants produced significantly larger number of axillary bud as compared to apical bud. Hence, axillary bud can be successfully utilized for initial culture establishment. Earlier, Xu *et al*. (11) successfully propagated cytoplasmic male sterile (CMS) lines of Chinese cabbage using axillary bud explants with a proliferation coefficient of 4.58. Studies by Zhang *et al.* (13) also revealed that axillary bud propagated faster than the hypocotyl during culture initiation.

The regenerated/sprouted shoots from different explants were dissected and inoculated to shoot multiplication media. Most of the micro-shoots placed on MS medium devoid of hormones remained as such or few shoots proliferated into two or three shoots thus producing on an average of 1.11 ± 0.19 shoots per micro-shoot. Significant improvement in shoot proliferation was observed with the addition of cytokinins (Table 2). Maximum shoot proliferation (5.89 ± 0.38) was obtained on MS medium supplemented with 5 mg l^{-1} KIN + 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} GA_3 (Fig. 1b). Among the three BA levels tested, best results

Table 1. Effect of explant types on *in vitro* culture establishment in cabbage.

Explant	Culture establishment (%)	Days to shoot sprouting	No. of shoots/ explant
Apical bud	83.33 ± 5.77[a]	9.07 ± 1.21[b]	7.20 ± 1.60[a]
Axillary bud	83.33 ± 5.77[a]	10.33 ± 0.58[b]	6.33 ± 0.64[a]
Basal shoot sprout	43.33 ± 11.54[b]	16.33 ± 1.70[a]	3.73 ± 1.50[b]

*Means followed by different letters within columns are significantly different at P ≤ 0.05, by Duncan's Multiple Range Test

Table 2. Effect of cytokinins on shoot proliferation in cabbage.

Treatment	KIN (mg l^{-1})	BA (mg l^{-1})	No. of shoots	Shoot length (cm)
T_1 (control)	-	-	1.11± 0.19^{f}	2.48 ± 0.15^{bc}
T_2	-	1.00	2.00 ± 0.33^{e}	1.97 ± 0.19^{d}
T_3	-	3.00	3.44± 1.02^{c}	2.17 ± 0.17^{cd}
T_4	-	5.00	2.56± 0.20^{de}	2.11 ± 0.13^{cd}
T_5	1.00	-	3.33± 0.11^{cd}	2.18 ± 0.28^{cd}
T_6	2.00	-	4.78± 0.50^{b}	2.50 ± 0.15^{bc}
T_7	3.00	-	3.22±0.18^{cd}	2.89 ± 0.25^{a}
T_8	5.00	-	5.89± 0.38^{a}	2.29 ± 0.39^{cd}
T_9	10.00	-	2.89± 0.51^{cd}	2.77 ± 0.20^{ab}

*Means followed by different letters within columns are significantly different at P ≤ 0.05, by Duncan's Multiple Range Test

were obtained with 3 mg l^{-1}. Xu *et al*. (11) also reported MS supplemented with BA and NAA at 2 and 0.4 mg l^{-1}, respectively as the most suitable medium for *in vitro* propagation of CMS Chinese cabbage, a drastic reduction in average rate of shoot proliferation was noticed at higher concentration of cytokinins. Profuse callus formation was observed when higher level of cytokinin was used, which might have reduced the rate of proliferation. The maximum shoot length (2.89 ± 0.25 cm) was recorded on MS medium was containing 3 mg l^{-1} KIN. The presence of cytokinins in the medium markedly increased the number of shoots produced per explant in rapid *in vitro* cloning of *B. oleracea* (Cheng *et al*., 2). The positive effect of cytokinins especially BA on regeneration from wide range of explants has been reported in different *Brassica* species (Yang *et al*., 12). We found that rate of proliferation was influenced by cytokinin type and concentration, with kinetin being more effective than BA. The efficiency of KIN for induction of multiple shoots in cauliflower have been earlier proved by Raut and Mahorkar (8). In contrary to the present studies KIN was found less effective for shoot multiplication using cotyledon and hypocotyl explants in cabbage (Munshi *et al*., 6). These differences in growth response may be attributed to difference in rate of metabolism of BA and kinetin. The shoots developed on media containing higher level of cytokinin were lanky and abnormal with linear strap-shaped leaves and exhibited vitrification symptoms. Vitrification was more prominent in BA supplemented media than KIN. An excess of cytokinins along with the high water potential of the medium are the major reasons for the vitrification of shoots. BA containing media caused a high incidence of hyperhydricity in the shoots, especially in the cauliflower and Savoy cabbage cultures. This problem can be overcome by the substitution of BA with KIN in the media (Suzana *et al*., 9).

Significant elongation of cabbage micro-shoots was observed when they were transferred to elongation medium supplemented with GA_3 (Fig. 2).

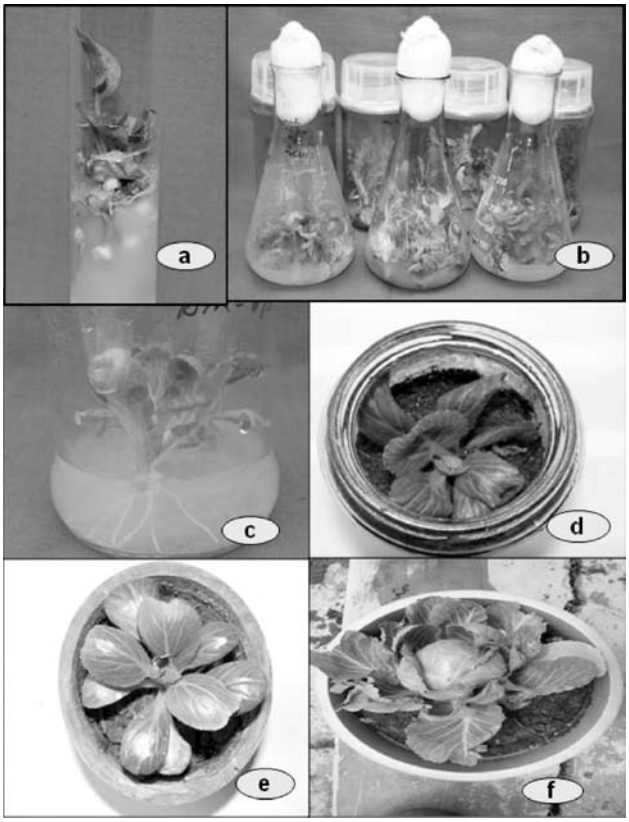

Fig. 1. *In vitro* culture establishment, shoot proliferation, rooting and hardening in cabbage. (a) Regeneration from apical bud, (b) Shoot proliferation, (c) Rooting of *in vitro* shoots, (d) Hardening of *in vitro* raised plants in glass jars, (e) *In vitro* raised hardened plants growing under outdoor conditions, and (f) Head formation on *in vitro* raised plant.

Gibberellins are known for inducing stem length in number of crops. Longest shoots (5.33 cm) were obtained on MS medium supplemented with 1.0 mg l^{-1} GA_3. Minimum shoot length (2.73 cm) was recorded on MS medium devoid of GA_3. The well-developed *in vitro* shoots approximately 5 cm in height were transferred on to half-strength MS medium containing different levels of IBA and NAA either singly or in combination (Table 3). The shoots cultured on medium devoid of auxins showed poor rooting (23.33 ± 5.77%) and took maximum time (27.22 ± 1.89 days) for root initiation. However, more than 60 per cent shoots initiated roots when auxin (IBA or NAA) was added into the medium. The earliest root induction (10.33 ± 0.33 days) was observed on half-strength MS medium containing 1.0 mg l^{-1} IBA. The maximum number of roots (8.33 ± 0.33) per shoot was recorded with 1.0 mg l^{-1} IBA. Wu *et al*. (10) also reported a rhizogenesis rate of 86.7% with 13.5 roots per shoot in broccoli on half-strength MS medium supplemented with 0.2 mg l^{-1} NAA.

Of the two auxins tested, IBA was found better over NAA for inducing good quality roots in cabbage. The roots on IBA supplemented media were long, well developed with root hairs and also uniformly distributed. The roots initiated on NAA supplemented media were short, stumpy and without root hairs. Earlier studies by Munshi *et al.* (6) have also proved the efficiency of IBA in cabbage. The synergistic effect of was observed for days to root initiation and number of roots per shoot only when both the auxins were used at low concentration. Higher concentration of either of NAA and IBA or their combination resulted in delayed rooting and significant reduction in number of roots. This may be due to the reason that the cut

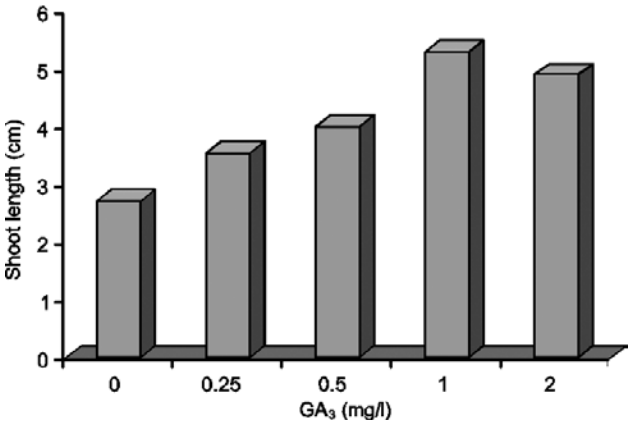

Fig. 2. Effect of GA_3 on elongation of cabbage micro-shoots.

end of micro-shoots initiated callus in which delayed rhizogenesis. Two hardening strategies, *viz*., glass jars with polypropylene caps and plastic pot with polythene cover were employed for hardening. Out of them, hardening in glass jars with polypropylene cap gave the best results (Fig. 1d). The high success in glass jar might be due to high moisture retention and also due to constant maintenance of relative humidity. The tissue cultured plants were successfully transferred to outdoor field condition with a survival rate of 76.67 per cent. A survival rate up to 85% after hardening of test tube plantlets have earlier been reported in broccoli (Wu *et al*., 10). Successful head formation, bolting and flowering was observed in these plants (Fig. 1e & f).

The developed protocol can be useful for the production high purity F_1 hybrid seed production and

Table 3. Effect of auxin(s) on rooting of cabbage micro-shoot.

Treatment	IBA (mg l^{-1})	NAA (mg l^{-1})	Rooting (%)	Days to rooting	No of roots/ shoot
T_1	-	-	23.33 ± 5.77^{e}	27.22 ± 1.89^{a}	1.11 ± 0.65^{f}
T_2	0.5	-	93.33 ± 11.54^{ab}	15.56 ± 0.84^{de}	5.74 ± 0.52^{c}
T_3	1.0	-	100.00 ± 0.00^{a}	10.33 ± 0.33^{g}	8.33 ± 0.33^{a}
T_4	2.0	-	86.67 ± 5.77^{b}	16.74 ± 1.32^{cd}	4.82 ± 0.73^{d}
T_5	-	0.5	76.67 ± 5.77^{c}	16.37 ± 0.61^{d}	4.52 ± 0.17^{d}
T_6	-	1.0	100.00 ± 0.00^{a}	13.89 ± 0.69^{e}	7.22 ± 0.51^{b}
T_7	-	2.0	83.67 ± 5.77^{bc}	21.11 ± 2.67^{b}	3.12 ± 0.39^{e}
T_8	0.25	0.25	96.67 ± 5.77^{ab}	14.67 ± 1.01^{de}	6.89 ± 0.38^{a}
T_9	0.5	0.5	100.00 ± 0.00^{a}	12.11 ± 1.73^{fg}	7.78 ± 0.50^{ab}
T_{10}	1.0	1.0	63.33 ± 5.77^{d}	18.67 ± 0.88^{c}	3.23 ± 0.39^{e}

*Means followed by different letters within columns are significantly different at P ≤ 0.05, by Duncan's Multiple Range Test

also eliminate labour intensive bud pollination for maintenance of parental line. This protocol can also be utilized in developing new lines and cultivars in shorter time.

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