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RESEARCH PAPER

Effect of Plant Growth Regulators and explants on efficient *in vitro* Regeneration of Broccoli (*Brassica oleracea* L. var. *italica*)

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ABSTRACT

An efficient *in vitro* regeneration protocol is a crucial tool in rapid production of desirable and essentially genetically identical plants and genetic engineering of the crop for improved characteristics. To establish a highly efficient, reliable and stable regeneration protocol for Broccoli (*Brassica oleracea* L. var. italica) several vital factors, like optimum concentration of appropriate growth regulators and explants, are very essential to be standardized. For this, hypocotyl and apical meristems were used as explants and cultured in MS medium incorporated with different hormonal combinations for callus induction (0.5, 1.0, 1.5, 2.0 mg/L NAA with 1.0 mg/L 2,4-D), shoot regeneration (1.5, 2.5, 3.5, 4.5 mg/L BAP with 0.5 mg/L NAA) and root initiation (0.20, 0.30, 0.5, 1.0 mg/L IBA). Among these concentrations, MS medium with 1.0 mg/L 2,4-D and 1.0 mg/L NAA was found best for callus initiation (72.5%) with average callus size of 7.32 cm^2 and average callus weight of 3.52 g within 15 days from apical meristem. The MS medium with 3.5 mg/L BAP and 0.5 mg/L NAA showed the best shoot regeneration (62.5%), average no. of shoots (6.03) with mean shoot length (4.90 cm) within 45 days. The regenerated shoots were rooted well in MS medium containing IBA (0.20 mg/L) with highest root initiation (90%), average no. of roots (6.28) and mean root length (4.63 cm) within 15 days. The survival rate of well rooted plantlets in the soil was 90%. Thus, it can be concluded that apical meristem was the best explant for efficient *in vitro* regeneration of Broccoli in the above mentioned hormonal combinations and concentrations.

Key words: Broccoli, Plant Growth Regulators, Explants, Regeneration.

Introduction

Broccoli (*Brassica oleracea* L. var. italica) is an economically important and highly diversified group of crops belonging to the family Brassicaceae. *B. oleracea* is one of the major species of this group which includes many distinct vegetable and fodder varieties, such as cabbage, broccoli, Brussels sprouts, cauliflower, collards, Savoy cabbage, kohlrabi, rutabaga and turnip. These are consumed worldwide as food of high nutritional value. *Brassicaceae* vegetables represent an important part of the human diet worldwide and are considered important food crops in China, India, Japan and European countries. Broccoli is rich in vitamin A, ascorbic acid and appreciable amounts of thiamin, riboflavin, niacin, calcium, iron (Thompson and Kelly, 1957; Lincolin, 1987) and medicinally important anti-cancerous compound sulphoraphane with potential application in the pharmaceutical industry. Broccoli has antimicrobial (Survay *et al.*, 2012; Jaiswal et al., 2011), antioxidant (Mahn and Reyes, 2011) and anticancer (Vasanthi *et al.*, 2009; Fahey *et al.*, 1997) activities. The Anti-microbial potential of broccoli extracts against food borne bacteria has potential application in food industries as botanical preservatives (Sibi et al., 2013). It is cleared that broccoli has high important value in daily intake.

Plant diseases, insects and other stressors can cause enormous yield reductions during commercial broccoli cultivation (Yang *et al*., 2002; Viswakarma *et al.*, 2004). Because of their significant importance *Brassica* vegetables are the objects of many breeding programs with the aimed at additionally improving the agronomic and nutritional performances of the existing genotypes. Nowadays, many breeders attempt to improve *Brassica* crops by employing the biotechnological and genetic

transformation approaches, in addition to the classic ones. *Brassica spp.* are generally considered to be recalcitrant in tissue culture. However, there are several reports regarding cabbage, canola, and broccoli transformation (Sretenović-Rajičić *et al.,* 2006; Sretenović-Rajičić *et al.,* 2007; Bhala and Singh, 2008). Also, *in vitro* regeneration from different explants *via* organogenesis has been achieved (Munshi *et al.,* 2007; Pavlović *et al.,* 2010). So far, with respect to cabbage, shoot regeneration has been achieved from various tissues and organs, including hypocotyls, cotyledons, roots, leaves, peduncle segments, callus and cell cultures, thin cell layers, protoplasts, and immature zygotic embryos (Cardoza and Stewart 2004; Kiełkowska and Adamus 2012; Pavlović *et al.*2013; Ravanfar *et al.*2014). A range of studies have noted substantial variation even if the same species or cultivar were investigated. *Brassica oleracea* subsp. *italica* is one of the many valuable *Brassica* species, which is still less cultured under *in vitro* condition (Widiyanto and Erytrina, 2001). Therefore, we tried to investigate the shoot-regeneration ability in *B. oleracea* var. *italica* that could be a prospective material for further transformation and somaclonal variation based breeding.

In most *Brassica* species, the successful application of *in vitro* culture is mostly dependent on the genotype and the influence of plant growth regulators (PGRs). The addition of cytokinins, such as kinetin or benzyladenine would enhance shoot proliferation and root formation (Arnison et al., 1990). Various concentrations of auxins such as naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and indoleacetic acid (IAA) have been evaluated for rooting of *in vitro* regenerated shoots of broccoli and cauliflower (Vandemoortele et al., 1999; Widiyanto and Erytrina, 2001). This paper reports on the influence of BAP in combination with NAA, on adventitious shoot proliferation from hypocotyls and epical meristem explant of broccoli cv. Green Marvel, and the effect of various concentrations of auxins such as indolebutyric acid (IBA) on rooting of the *in vitro* regenerated shoots. Therefore, the aims of the present study were to find out suitable explant and, concentrations and combinations of plant growth regulators and to establish a highly efficient, reliable and reproducible regeneration protocol for Broccoli.

Materials and methods:

Plant material and sterilization protocol:

Seeds of broccoli were surface sterilized for 2 min in 70% ethanol solution followed by rinsing three times in sterile distilled water and continuous agitation in 6% sodium hypochlorite for 15 min and rinsing three times in sterile distilled water. The seeds were placed in sterilized tissue paper to remove excess water from the surface of the seeds and cultured on MS (Murashige and Skoog,1962) medium containing half-strength MS salts supplemented with 8 g/L agar and 30 g/L sucrose. Explants were collected from 7 days old broccoli seedlings. The apical meristem and hypocotyl of the experimental plants were used as the explants.

Medium composition and treatment:

The following concentrations and combinations of plant growth regulators were used for the present investigation those were as follows:

1) For callus induction: MS medium supplemented with 0.5, 1.0, 1.5, and 2.0 mg/L NAA in combination with 1.0 mg/L2, 4-D.

2) For shoot regeneration: MS medium supplemented with 1.5, 2.5, 3.5, and 4.5 mg/L BAP in combination with 0.5 mg/L NAA.

3) For root initiation: MS medium supplemented with 0.20, 0.30, 0.50, 1.0 mg/L IBA.

Parameters recorded

In case of callus induction from hypocotyl and apical meristem explants parameters were recorded. Days required for callus initiation, Size of callus, Weight of callus, Percentage of callus initiation. The observations of explants on culture media were started from 7th day of inoculation and continued up to 45 days. In the shoot induction and multiplication, parameters recorded were Days required for shoot initiation, Average number of shoot per callus, Shoot length (cm), Percentage of shoot regeneration. The observations of shoot regeneration media were started from 24th day of inoculation and continued up to 90 days. In the rooting study, the parameters recorded were Days required for root initiation, Average number of roots per shoot, Root length (cm), Root initiation percentage (%). The data for root initiation were started from 7th day after inoculation and the data were recorded on a weekly basis up 25th day. The experiments were arranged in a Completely Randomized Design (CRD), with four replications and each replication per treatment contained 10 explants. The data of all parameters were statistically analyzed by using Minitab 17 package program. Recorded data were analyzed statistically using analysis of variance technique (ANOVA) and means were compared by Duncan's Multiple Range Test at 5% level of probability.

Results and discussion

Response of explants at different concentrations of NAA with 1 mg/L 2, 4-D for induction of callus

Hypocotyls and apical meristems were excised from 7-day-old broccoli seedlings and cultured on callus induction media to observe callusing response (Table 1). Hypocotyl (80%) and apical meristem (90%) both explants showed the best callusing response in a combination of 1.0 mg/L NAA with 1.0 mg/L 2,4-D among all the combinations and concentrations of PGRs. According to Ravanfar *et al.* (2011), the media with low concentration of NAA (0.5 mg/L) showed better callus formation. Apical meristem (72.5%) showed better average responses of callus induction than hypocotyl in all the combinations and concentrations of PGRs. The probable reason of such result would be the totipotent capacity of the meristematic cells of the apical meristem explant.

Effect of explants and hormonal concentrations on size and weight of callus

The size and weight of callus from both explants were recorded (Table 2). In case of size and weight both parameters,

hypocotyl (size 3.52 cm^2 and weight 1.96 gm) and apical meristem (size 7.32 cm^2 and weight 3.52 gm) both explants showed the best results in the combination of 1.0 mg/L NAA with 1.0 mg/L 2,4-D among all the combinations and concentrations of PGRs applied. According to Ravanfar *et al.* (2011), the medium with low concentration of NAA showed better callus formation. The apical meristem showed better average responses for both callus size (4.56 cm^2) and weight (2.31 gm) than the hypocotyl considering all the combinations and concentrations of PGRs (Fig. 1). Pictorial views of the calli induced in different treatments are shown in figure 2. The probable reason of such result would be the totipotent capacity of the meristematic cells of the apical meristem explants.

Table 1. Response of explants at different concentrations of NAA with 1 mg/L 2,4-D for induction of callus

Explant	Supplements (mgL^{-1})		No. of explants inoculated	No. of explants showing callus	Callus induction (%)	Average of callus induction (%)
	2,4-D	NAA				
Hypocotyl	1.0	0.5	10	5	50	60
		1.0	10	8	80	
		1.5	10	7	70	
		2.0	10	4	40	
Apical meristem	1.0	0.5	10	7	70	72.5
		1.0	10	9	90	
		1.5	10	8	80	
		2.0	10	5	50	

Note: Days required for callus induction in all the treatments were 15.

Table 2. Effect of explants and hormonal concentrations on size and weight of callus

Explant	Supplements (mgL^{-1})		Size of callus (cm^2)	Weight of callus (gm)
	2,4-D	NAA		
Hypocotyl	1.0	0.5	1.78c	1.34c
		1.0	3.52a	1.96a
		1.5	2.88b	1.72b
		2.0	1.56d	1.05d
Apical meristem	1.0	0.5	3.39c	1.87c
		1.0	7.32a	3.52a
		1.5	6.30b	2.88b
		2.0	1.22d	0.96d

Note: Mean values having common letter(s) are statistically identical and those having different letter(s) are statistically different (for each explant separately).

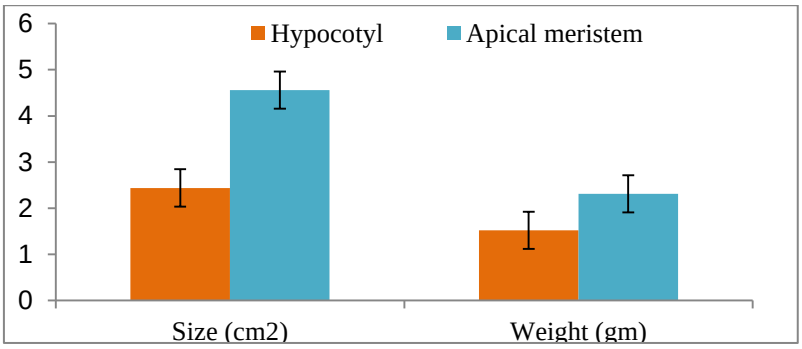

Fig. 1. Average size and weight of calli induced from hypocotyl and apical meristem cultured in MS media supplemented with different combinations and concentrations of 2,4-D and NAA. Error bar represents standard error of mean among the mean values of size and weight of callus.

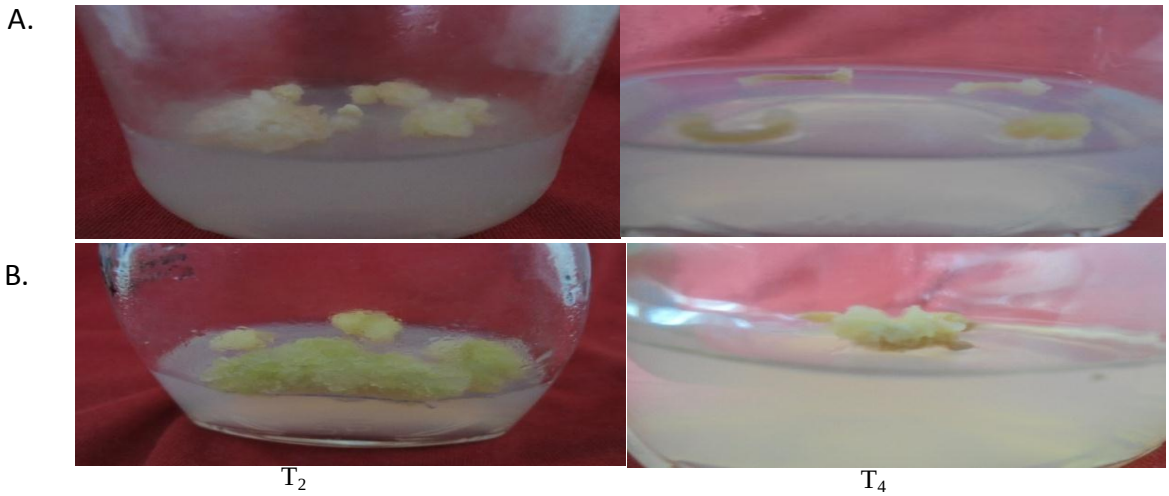

Fig. 2. Callus induction from A) hypocotyl and B) apical meristem explants in T_2= 2, 4-D 1.0 mg/L + NAA 1.0 mg/L and T_4= 2, 4-D 1.0 mg/L + NAA 2.0 mg/L PGR combination.

Response of callus induced from various explants at different concentrations of BAP with 0.5 mg/L NAA for shoot regeneration

The calli induced from both hypocotyl and apical meristems were cultured in shoot initiation media to observe regeneration performance of these calli (Table 3). Among all combinations and concentrations BAP (3.5 mg/L) with NAA (0.5 mg/L) showed the best shoot regeneration percentage in case hypocotyl (70%) and apical meristem (80%) as well. Apical meristem (62.5%) showed better average responses of shoot initiation than hypocotyl (55%) considering all the combinations and concentrations of PGRs. Ravanfar *et al.* (2011) also found better shoot regeneration percentage using combination of BAP and NAA.

Effects of explants and hormonal concentrations on number and length of shoots

The no. of shoots per callus induced from both explants with mean shoot lengths was recorded and the results are presented in table 4. In number and length both cases of shoots, calli induced from hypocotyl (number 5.76 and length 4.29 cm) and calli induced from apical meristem (number 6.03 and length 4.90 cm) both parameters showed the best results in the combination of 0.5 mg/L NAA with 3.5 mg/L BAP among all the combinations and concentrations of PGRs applied. It has been previously shown that BAP either alone or in combination with auxin to be optimal for shoot regeneration and multiplication in different Brassica species (Metz *et al*., 1995; Jin *et al*., 2000; Munshi *et al*., 2007; Sretenović-Rajičić *et al*., 2007; Maheshwari *et al*., 2011). Addition of BAP in the medium significantly increased the number of shoots per explant in rapid cycling *B. oleracea in vitro* (Cheng *et al*., 2001). Furthermore, the supplementation of NAA was shown to significantly enhance shoot regeneration (Guo *et al*., 2005). Therefore, in this study, we used the media containing BAP in combination with NAA. The calli induced from apical meristem showed better average responses for number (4.54) and length (3.76 cm) of shoots compared to the calli induced from hypocotyl explant considering all the combinations and concentrations of PGRs (Fig. 3). Pictorial views of the shoots initiated from different treatments are shown in figure 4. Young explants with more meristematic cells have been shown to give better regeneration response than older explants in most *Brassica* species (Chakrabarty *et al*., 2002; Sharma *et al*., 2012; Sharma *et al*., 2014). A possible explanation is that young apical meristem explants are physiologically and biochemically more active as they have less rigid cell wall and are easily affected by the environmental factors such as exogenous plant growth regulators.

Response of shoots initiated from calli of various explants at different concentrations of IBA for initiation of roots

Successful rooting of *in vitro*-derived shoots is an integral part of each regeneration protocol. To achieve this, the regenerated shoots from the calli of both hypocotyl and apical meristems explants were cultured in root initiation media to see their rooting responses (Table 5). Shoots regenerated from calli of both the explants showed the best root initiation in 0.20 mg/L IBA among all the concentrations of IBA investigated. Only in case of rooting, shoots of hypocotyl explants (70%) showed better performance than the shoots of apical meristem explants (62.5%). According to Ravanfar *et al.* (2011), the shoots were subsequently rooted in MS medium that contained 0.2 mg/L of IBA.

Effects of explants and hormonal concentrations on average number and length of roots

The no. of roots per shoot with mean root lengths were recorded and the results were presented in table 6. In number and length both cases of roots, shoots

Table 3. Response of calli induced from various explants at different concentrations of BAP with 0.5 mg/L NAA for shoot regeneration

Explant	Supplements (mgL^{-1})		No. of callus inoculated	No. of callus showing shoot regeneration	Shoot regeneration (%)	Average shoot regeneration (%)
	NAA	BAP				
Hypocotyl	0.5	1.5	10	4	40	55
		2.5	10	5	50	
		3.5	10	7	70	
		4.5	10	6	60	
Apical meristem	0.5	1.5	10	5	50	62.5
		2.5	10	5	50	
		3.5	10	8	80	
		4.5	10	7	70	

Note: Days required for initiating shoot in case of all the treatments were 45.

Table 4. Effects of explants and hormonal concentrations on average number and length of shoots

Explant	Supplements (mgL^{-1})		No. of shoot per callus	Length of shoot (cm)
	NAA	BAP		
Hypocotyl	0.5	1.5	1.92d	1.53d
		2.5	2.05c	1.86c
		3.5	5.76a	4.29a
		4.5	4.90b	3.94b
Apical meristem	0.5	1.5	2.97d	2.59d
		2.5	3.92c	3.51c
		3.5	6.03a	4.90a
		4.5	5.23b	4.03b

Note: Mean values having common letter(s) are statistically identical and those having different letter(s) are statistically different (for each explant separately).

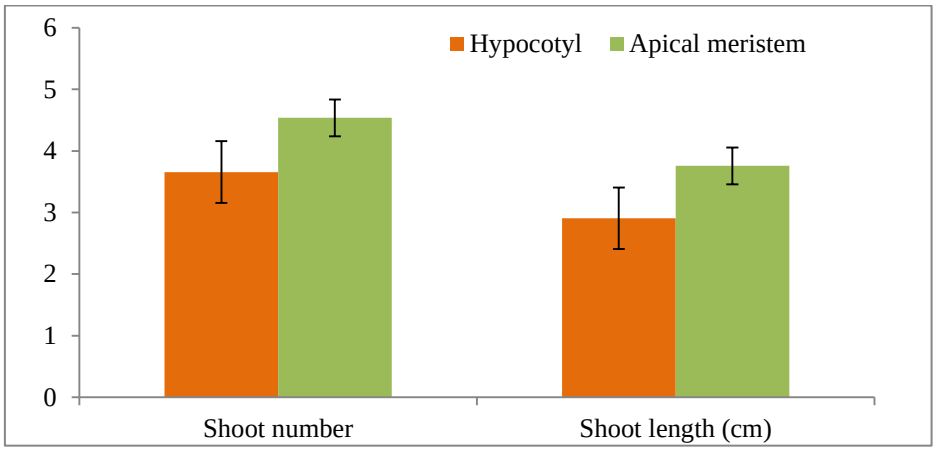

Fig. 3. Average number and length of shoots regenerated from calli of hypocotyl and apical meristem explants cultured in MS media supplemented with different combinations and concentrations of NAA and BAP.
Error bar represents standard error of mean among the mean values of number and length of shoots.

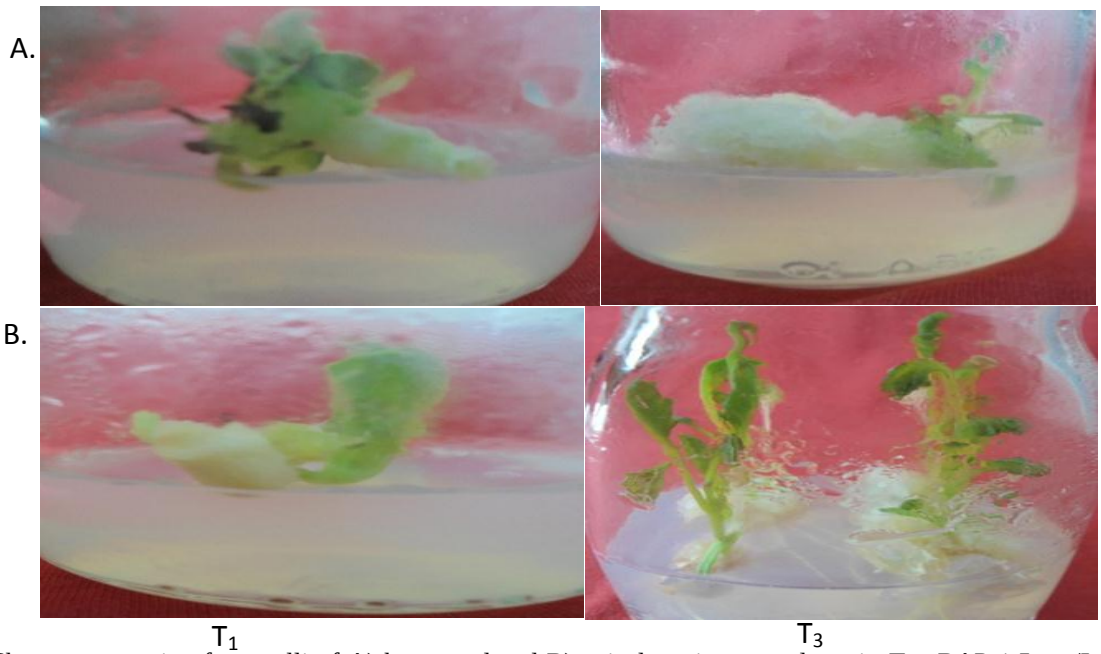

Fig. 4. Shoot regeneration from calli of A) hypocotyl and B) apical meristem explants in T_1= BAP 1.5 mg/L + NAA 0.5 mg/L and T_3= BAP 3.5 mg/L + NAA 0.5 mg/L PGR combinations.

regenerated from calli of hypocotyl (number 6.03 and length 4.37 cm) and calli induced from apical meristem (number 6.28 and length 4.63 cm) both parameter showed the best results in 0.2 mg/L IBA among all the concentrations of IBA applied. Ravanfar *et al.*, 2009 and Sharma *et al.*, 2014 reported that medium containing IBA was most suitable for root regeneration. Nevertheless, the influence of NAA, IAA or IBA on root induction was highly dependent on genotype (Arnison *et al.*, 1990; Vandemoortele *et al.*, 1999). Average responses for number and length of roots initiated from the shoots of the calli of both explants considering all the concentrations of IBA are not significantly different. Pictorial views of the roots initiated from different treatments are shown in figure 5.

Table 5. Response of shoots initiated from calli of two explants at different concentrations of IBA for initiation of roots

Explant	Supplement (mg L^{-1}) IBA	No. of shoots incubated	No. of shoots showing root initiation	Root initiation %	Average root initiation %
Hypocotyl	0.20	10	9	90	70
	0.30	10	6	60	
	0.50	10	8	80	
	1.0	10	5	50	
Apical meristem	0.20	10	9	90	62.5
	0.30	10	5	50	
	0.50	10	7	70	
	1.0	10	4	40	

Note: Days required for root initiation in all the treatments were 15.

Table 6. Effects of explants and hormonal concentrations on average number and length of roots

Explant	Supplement (mg/L) IBA	No. of roots per shoot	Length of root (cm)
Hypocotyl	0.20	6.03a	4.37a
	0.30	4.27c	3.90c
	0.50	5.39b	4.09b
	1.0	2.98d	1.93d
Apical meristem	0.20	6.28a	4.63a
	0.30	3.92c	2.58c
	0.50	5.97b	4.18b
	1.0	2.10d	1.70d

Note: Mean values having common letter(s) are statistically identical and those having different letter(s) are statistically different (for each explant separately).

Fig. 5. Root initiation from regenerated shoots derived from calli of A) hypocotyl and B) apical meristem explant in T_1= 0.2 mg/L IBA and T_4= 1.0 mg/L IBA.

Acclimatization of Plants

The plantlets with sufficient roots were taken out from the culture vessels and thoroughly washed in running tap water to remove all adherent culture medium. The plantlets were then transplanted to small pots covering with polybags to maintain moisture for 7 to 15 days (Fig. 6). The survival rate of plantlet under natural environment was 90%. A 100% survival rate of the regenerants of broccoli in natural environment was obtained by Handayani (2014).

Fig. 6. Acclimatization of regenerated plantlets in normal environmental condition.

Summary and Conclusions

Among all combinations and concentrations 1.0 mg/L NAA in combination with 1.0 mg/L 2, 4-D showed the best callusing response with both hypocotyl (80%) and apical meristem (90%) explants where apical meristem (72%) showed best average performance considering all the combinations and concentrations of NAA and 2, 4-D. The best mean callus size (3.52 cm^2) and mean callus weight (1.96 g) obtained from hypocotyl and the best mean callus size (7.32 cm^2) and mean callus weight (3.52 g) obtained from apical meristem were observed in the same combination of NAA and 2,4-D. Considering all the combinations and concentrations of NAA and 2,4-D the average callusing response was better from apical meristem explants. For shoot regeneration, among all combinations and concentrations, 3.5 mg/L BAP with 0.5 mg/L NAA showed the best shoot regeneration from calli of hypocotyl (70%) and apical meristem (80%) explants. The highest no. of shoots per callus (5.76) with mean shoot length (4.29 cm) obtained from hypocotyl and the highest no. of shoots per callus (6.03) with mean shoot length (4.90 cm) obtained from apical meristem. Here also calli of apical meristem explants showed the best average performance considering all the combinations and concentrations of NAA and BAP. For root initiation, among all concentrations IBA (0.20 mg/L) showed better root initiation from both hypocotyl (90%) and apical meristem (90%). The highest no. of roots per plant (6.03) with mean root length (4.37 cm) obtained from hypocotyl and the highest no. of roots per plant (6.28) with mean root length (4.63 cm) obtained from apical meristem. But here shoots regenerated from the calli of hypocotyl explants showed the best average performance considering all the combinations and concentrations of IBA.

From the above results of this research work, it may be concluded that the MS medium containing 1.0 mg/L 2, 4-D and 1.0 mg/L NAA was found suitable for callus induction from both hypocotyl and apical meristem. MS medium supplemented with 3.5 mg/L BAP with 0.5 mg/L NAA found best for shoot regeneration from callus of hypocotyl and apical meristem. MS medium supplemented with 0.2 mg/l IBA was the best treatment for root induction. Apical meristem was found the best explant for *in vitro* regeneration of broccoli.

Further comparative study is needed to test the field performance of tissue culture derived broccoli plantlets. The protocol developed through tissue culture method from this research would be helpful for the production of more number of healthy and disease free plantlets and extend the demand and availability of broccoli in Bangladesh.

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