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***In vitro* shoot proliferation and development of micropropagation protocol from leaf disc of Gladiolus**

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Abstract

The experiment was carried out during the period from July 2008 to March, 2009 at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh. The present investigation was to be better source for shoot multiplication. The calli derived from leaf discs were cultured on shoot induction media containing different combinations and concentrations of BAP (1.0, 1.5, 3.0 and 6.0 mg/L) and Kn (0.5, 1.0, 1.5 and 2.0 mg/L). The highest percentage of shoot regeneration (91.66%) and the highest shoot length was 4.24 (cm) with a minimum number of days (13.33) was observed in the MS medium supplemented with 3.0 mg/L BAP and 0.5 mg/L Kn. Thus shoot multiplication in successive subculture was possible. The present investigation was carried out to study the *in vitro* shoot multiplication in gladiolus by using leaf disc as explants

Keyword: Shoot proliferation, Micropropagation, BAP: 6-benzylamino purine, Kn: Kinetin, Leaf disc

Introduction

Gladiolus perennates by underground stems or corms, the infection by soil-borne fungus, *Fusarium oxysporum* f. sp. *gladioli* causing severe loss and damage is a major bottleneck to its mass propagation. With the growing symbiosis between plant breeders and biotechnologists, disease resistant varieties of Gladiolus developed through plant transformation will be a reality in near future and some reports are already available (Kamo, 1997; Kamo and Blowers, 1999; Loffler *et al.*, 2000). It propagates either by seeds, corm formation or by Cormel differentiation. Although, seeds are an effective means of Gladiolus propagation but seed-raised plants may not produce true-to-type population (Hussain *et al.*, 2001). Moreover, it takes four seasons for blooming. The propagation by corm and cormel formation is another conventional method of multiplication but it may transmit several viral, fungal and bacterial diseases e.g., *Fusarium* corm rot, *Botrytis* blight, bacterial leaf rot etc., thus causing a heavy loss.

Plant tissue culture offers a potential to deliver large quantities of disease-free, true-to-type healthy stock within a short span of time (Hussain *et al.*, 2001). The *In vitro* multiplication of Gladiolus has been achieved by using axillary buds (Boonvanno and Kanchanapoom, 2000; Begum and Haddiuzzaman, 1995), shoot tip (Hussain *et al.*, 2001), cormels (Nagaraju and Parthasarathy, 1995) and inflorescence axes (Ziv and Lilien-Kipnis, 2000). Moreover, successful protocols for *In vitro* corm formation (Dantu and Bhojwani, 1995; Sen and Sen, 1995 and Al-Juboory *et al.*, 1997), organogenesis and somatic embryogenesis (Remotti and Toffler, 1995; Kumar *et al.*, 2002) have also been achieved. However, in Gladiolus there is a clear scope for further refinement through *In vitro* culture methodology to acquire a higher number of shoots to complement traditional nursery methods (Hussain *et al.*, 2001). Based on the above information, the present study was undertaken within the following objectives-

- To determine a suitable hormone combination shoot regeneration and plantlet establishment; and
- To develop protocol for *in vitro* plantlet regeneration of Gladiolus

Materials and Methods

The experiment was carried out during the period from July 2008 to March, 2009 at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh. The planting materials (bulbs) of Gladiolus from four varieties were collected from Germplasm Center, Bangladesh Agricultural Research Institute. The Bulbs were used to germinate plants *in vitro* condition and after three weeks, the leaves were used as explants. MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones as treatments was used in culture medium for shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoot. The hormones were

added separately to MS media. For the preparation of media, stock solutions were prepared at the beginning and stored at $4\pm1^{\circ}\text{C}$. The media were prepared from the stock solutions. The leaf derived calli were cultured on MS medium supplemented with combinations of various concentrations of BAP (1.0, 1.5, 3.0 and 6.0 mg/l) and Kn (0.5, 1.0, 1.5 and 2.0 mg/L) for shoot initiation.

For the purpose of hardening and acclimatization to the field conditions, 10 plants (8-10 weeks old) were taken out of the culture vessels. The roots of the plantlets were thoroughly washed with tap water in order to remove even the traces of the nutrient medium. The plantlets were then transferred to the pots containing presterilized sand. The pots were kept in the culture room for two weeks; adequate water was given to the plantlets at an interval of two-to three days. After two weeks, the plants were shifted to the soil in pots and kept in the growth room. After further hardening of two weeks, the plants were shifted to the normal growth conditions of the field.

In this study, an efficient and reproducible protocol for the plantlet regeneration from leaf discs explants in Gladiolus was developed. This protocol has the potential for the *in vitro* regeneration of Gladiolus.

Results and Discussion

For shoot regeneration, leaf derived calli were cultured on shoot induction media containing different combined concentrations of BAP and Kn. The basal most portions of the innermost leaves used in the present study were the most responsive to adventitious multiple shoot bud formation. Histological observation revealed direct organogenesis and nature of response (Fig. 1). Indirect mode of shoot organogenesis, via callusing through is in literature (Sinha and Roy, 2002; Kumar *et al.*, 1999) but the direct one as observed in the present study is definitely of advantage in case on maintaining clonal purity. The results on the effect of different concentrations of BAP and Kn for shoot initiation have been discussed bellow:

Days required for shoot initiation

Main effect of BAP (mg/L): Cultured explants were carefully observed in regular basis for days required for shoot regeneration. The growth regulator concentrations significantly affected the days to shoot initiation. Table 1 show that 3.0 BAP was the best and needed least number of days for shooting. Maximum numbers of days (21.66 days) were required in the medium containing 6.0 mg/l BAP. Hussain *et al.*, (2001) studied shoot multiplication of two varieties (White and pink) of gladiolus. They reported the maximum number of shoots from shoot tip explants at a much higher BAP concentrations (3.0mgL^{-1} and 4.0 mgL^{-1} for the two varieties). They suggested the higher dose requirement of BAP would be genotype-dependent. But Anandhi and Seker (2000) reported that the kinetin (4.0 mg/liter) without addition of auxin was found to be effective in hastening the time taken for direct organogenesis (12 days compared to BAP).

Main effect of Kn (mg/l): Cultured explants were carefully observed in regular basis for data collection on days required for shoot regeneration. The growth regulator concentrations significantly affected the days required for shoot initiation. Table 2 shows that 1.0 Kn was the best and needed the least number of days (18 Days) for shooting. Maximum numbers of days (19 Days) were required in the 2.0 mg/L Kn.

Combined effect of different concentration of BAP and Kn: The Combined effect of different concentration of BAP and Kn were significantly affected the days for shoot initiation. Table 3 shows that combined effect of 3.0 BAP and 0.5 Kn was better and needed the least number of days for shooting. Maximum numbers of days (23 days) were required in the 6.0 mg/L BAP and 2.0 mg/L Kn.

Percentage of explants induced shoot

Main effect of BAP (mg/L): The highest percentage of shoot initiation was (78.67%) observed with the supplementation with 3.0 BAP within a minimum number of days (16 days) were required. Faheem Aftab *et al.*, (2008) reported shoot induction was possible with a maximum rate (80%) at 1mg/l BAP by using meristem explants. The results therefore, nearly corroborate the findings of Nagaraju and Parthasarathy (1995). The lowest percentages of shoot were (26.53%) observed in the addition of medium without BAP (Table 1) within a minimum number of days (16) were required.

Table 1. Main effect of different concentrations of BAP on percent shoot proliferation, days required for shoot initiation and length of shoot (cm) of variety cv. GL4

BAP (mg/l)	Percent shoot regeneration	Days required for shoot initiation	Length of shoot (cm)
0	26.53 d	16.46	0.78 c
1.0	49.33 c	18.13	2.53 b
1.5	61.40 b	18.46	2.46 b
3.0	78.06 a	15.73	3.64 a
6.0	31.80 d	21.66	0.96 c
LSD(0.05)	3.05	0.597	0.35
CV (%)	6.11	3.25	17.07

Figure(s) within the same column bearing different letters are significantly different at 5% level according to DMRT

Main effect of Kn (mg/L): The highest percentage of shoot initiation was (55.33%) observed with the supplementation with 1.5 mg/L Kn within a minimum days was 18 and the lowest percentage of shoot was (35.80 %) observed in the addition of medium without Kn (Table 2) within a minimum days was 15.80. Anandhi and Sekar (2000) reported that the maximum (60%) response was obtained from shoot tip in the concentration of kinetin (4.0 mg/l) without addition of auxin. This does in agreement with the findings of Rao *et al.*, 1991.

Table 2. Main effect of different concentration of kinetin on percent shoot proliferation, days required for shoot initiation and length of shoot (cm) of variety cv. GL4

Kn (mg/l)	Percent shoot proliferation	Days required for shoot initiation	Length of shoot (cm)
0	35.80 c	15.80	1.76 b
0.5	47.26 b	18.60	2.10 ab
1.0	53.46 a	18.00	1.96 a
1.5	55.33 a	18.73	2.10 ab
2.0	55.26 a	19.33	2.45 a
LSD(0.05)	3.05	0.59	0.35
CV (%)	6.11	3.25	17.07

Figure(s) within the same column bearing different letters are significantly different at 5% level according to DMRT

Combined effect of different concentrations of BAP and Kn: The highest percentage of shoot initiation was (91.67%) observed with the combined supplementation with concentration of 3.0 mg/L BAP and 0.5 mg/L Kn within a minimum days were required (13days) and the lowest percentage of shoot was (17.67 %) observed in the addition of 6.0 mg/l BAP and 2.0 mg/L Kn (Table 3).

Length of shoot

Main effect BAP (mg/L): The concentration of 3.0 BAP was resulted the longest shoot (3.65 cm) and the shortest shoot was 0.79 cm was observed in medium without BAP (Table 1). Anandhi and Sekar (2000) reported that BAP 4.0 (mg/l) was obtained 2.5 cm length of shoot.

Main effect Kn (mg/L): The concentration of 1.0 mg/L Kn resulted the longest shoot (1.93 cm) and the shortest shoot (1.767 cm) was observed with supplementation of 0.5 mg/l Kn (Table 2). Anandhi and Sekar (2000) reported that shoot length was 3.0 cm in kinetin (4.0mg/L).

Combined effect of different concentrations of BAP and Kn: The concentration of 3.0 BAP and 0.50 mg/L Kn resulted the longest shoot (4.24 cm) and the shortest shoot (0.56 cm) was observed medium without of BAP and 0.5 mg/L Kn (Table 3).In order to increase the number of shoots per culture vessel, the explants were subcultured on the same medium after a regular interval of 28- 30 days. The calli initiated on different media were also maintained by sub culturing in the same way.

Conclusion

Studies were conducted with gladiolus in order to standardize the protocol for shoot proliferation *in vitro*. Shoot multiplication of *Gladiolus grandiflorus* was studied by using leaf disc as explants and achieved maximum shoot multiplication on MS Medium containing 3.0mg/L BAP and 0.5 mg/L Kn. Although it was difficult to quantify but we have observed that shoots obtained from leaf disc explants generally healthier (better vigour). So the leaf discs are a good source tissue for this purpose.

Table 3. Combined effect of different concentrations of BAP and Kn on percent shoot proliferation, days required for shoot initiation and length of shoot (cm) of variety cv. GL4

BAP (mg/l)	Kinetin (mg/l)	Percent shoot proliferation	Days required for shoot initiation	Length of shoot (cm)
0	0	0.00 p	0.00	0.00 k
	0.5	18.00 o	23.00	0.56 jk
	1.0	26.66 n	20.33	0.83 j
	1.5	38.00 jkl	19.66	0.90 j
	2.0	50.00 i	19.33	1.63 gh
1.0	0	19.00 o	21.66	1.73 gh
	0.5	27.33 mn	18.66	1.82 gh
	1.0	59.66 gh	17.33	2.13 efg
	1.5	65.66 ef	16.66	2.23defg
	2.0	75.00 cd	16.33	4.37 a
1.5	0	42.00 jk	19.66	2.12 efg
	0.5	56.00 h	17.66	2.92 c
	1.0	65.00 fg	17.33	1.92 fg
	1.5	73.33 cd	18.33	2.84 cd
	2.0	70.66 de	19.33	2.52 cdef
3.0	0	85.33 b	16.33	3.73 b
	0.5	91.66 a	13.33	4.24 a
	1.0	78.33 c	14.33	3.92 b
	1.5	72.00 d	16.33	3.60 b
	2.0	63.00 fg	18.33	2.72 cde
6.0	0	32.66 lm	21.33	1.23 hi
	0.5	43.33 j	20.33	1.00 hij
	1.0	37.66 kl	20.66	1.00 ij
	1.5	27.66 mn	22.66	0.95 ij
	2.0	17.66 o	23.33	0.65 j
LSD(0.05)		5.22	1.020	0.61
CV (%)		3.25	17.07	17.07

Figure(s) within the same column bearing different letters are significantly different at 5% level according to DMRT



Plate 1. Multiple shoots from leaf derived calli in MS medium supplemented with 3.0 mg/L



Plate 2. Multiple shoots from leaf derived calli in MS medium supplemented with 1.5 mg/L BAP and 1.0 mg/L Kn



Plate 3. Multiple shoots from leaf derived calli in MS medium supplemented with 6.0 mg/L BAP and 0.5 mg/L Kn

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