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IN VITRO SHOOT MULTIPLICATION AND CALLUS INDUCTION IN *GLADIOLUS HYBRIDUS* HORT.

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Abstract

The present investigation was carried out to study the *In vitro* shoot multiplication and callogenesis in *Gladiolus.* For this purpose, cormel, meristem and leaf explants were chosen. Different concentrations of BAP i. e., 0.5, 1.0, 1.5, 2.0 or 2.5 mgL^{-1} (2.22, 4.44, 6.66, 8.88 or 11.10 μM) were supplemented to MS medium. Both cormel and meristem explants showed maximum multiplication on MS medium containing $1mgL^{-1}$ BAP. At this particular concentration of BAP, 16 shoots (2 cm or longer) were developed from cormel while 10 from meristem explants after 8 weeks of culture initiation. Thus cormel explants proved to be a better source for shoot multiplication as compared to meristem. Further shoot multiplication in successive subcultures was also possible. Rooting of *In vitro* grown shoots was achieved on MS (half basal) medium supplemented with $0.5mgL^{-1}$ (2.69 μM) NAA or $2mgL^{-1}$ (9.84 μM) IBA.

Out of 14 tested media for callus induction, 3 media were found effective for callus induction from cormel explants. Only 2 media could support callus induction from leaf explants. Rooting was observed in one month old cormel-derived callus cultures when NAA was used at either 0.3 or 0.4 mgL^{-1} concentration (1.61 or 2.15 μM). Sporadic shoot regeneration was observed from cormel explant-derived callus cultures in MS medium supplemented with 2 mgL^{-1} (9.05 μM) 2, 4-D and 1.0 mgL^{-1} (4.44 μM) BAP.

Abbreviations: 2, 4-D: 2, 4-dichlorophenoxyacetic acid, BAP: 6-benzylamino purine, dicamba: 3, 6-dichloro-o-anisic acid, IBA: 3-indolebutyric acid, MS: Murashige and Skoog (1962) basal medium, NAA: Naphthaleneacetic acid, PGRs: Plant growth regulators.

Introduction

Gladiolus (Iridaceae) is a bulbous ornamental plant. It has a great commercial importance in cut flower industry all over the world as well as in Pakistan due to its magnificent and colorful spikes. 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 & Kanchanapoom, 2000; Begum & Haddiuzaman, 1995), shoot tip (Hussain *et al*., 2001), cormels (Nagaraju & Parthasarathy, 1995) and inflorescence axes (Ziv & Lilien-Kipnis, 2000). Moreover, successful protocols for *In vitro* corm formation (Dantu & Bhojwani, 1995; Sen & Sen, 1995 and Al-Juboory *et al*., 1997), organogenesis and somatic embryogenesis

(Remotti, 1995; Kumar *et al*., 2002) have also been achieved. However, in *Gladiolus* there is a clear scope for further refinement in *In vitro* culture methodology to acquire a higher number of shoots to complement traditional nursery methods (Hussain *et al.,* 2001).

The present study is an attempt to produce multiple shoots by using cormels and meristem as explants. It further compares callus induction response of cormel and leaf explants on different combinations of growth regulators.

Materials and Methods

Procurement and preparation of explant: The plant material was obtained from the Seed Center, University of the Punjab, Lahore. *Gladiolus* cormels prior to surface disinfestations were washed with detergent and tap water followed by 4-5 rinses with distilled water. The cormels of gladiolus have an outer brown protective covering that was removed before surface disinfestation. The surface disinfestation of explants was accomplished by dipping cormels in 0.6% (v/v) solution of commercial bleach (NaOCl) for 15-20 minutes with 0.1% Tween 20. Afterwards, cormels were rinsed several times (5-8 rinses) with autoclaved distilled water under a laminar airflow cabinet. Either whole or tip of the cormel (containing the meristematic tissue; 3 to 5 mm thick) was inoculated for micropropagation. For callogenesis, either leaves (1 to 1.5 cm^2) of *In vitro* grown plants or 2-3 mm thick slices of cormels were used as an explant.

Growth media: MS basal medium (Murashige & Skoog, 1962) at its half and full strength was used for shoot induction and proliferation. In addition, MS medium was also supplemented with 0.5, 1.0, 1.5, 2.0 or 2.5 mgL^{-1} BAP thus bringing the total combinations to 7. For the rooting of *In vitro* shoots, half strength MS medium was either supplemented with 2mgL^{-1} IBA or 0.5mgL^{-1} NAA (two combinations). For rooting, 2 cm or longer *In vitro* grown shoots were transferred to rooting medium.

To initiate callus from cormel and leaf explants, MS medium was supplemented with 0.3 to 4.0 mgL^{-1} NAA (seven combinations), 3 mg/l 2,4-D, 0.4 mgL^{-1} NAA + 0.5 mgL^{-1} BAP, 0.4 mgL^{-1} NAA + 1.0 mgL^{-1} BAP, 0.4 mgL^{-1} NAA + 3.0 mgL^{-1} BAP, 1.0 mgL^{-1} BAP + 0.2 mgL^{-1} 2,4- D, 1.0 mgL^{-1} BAP + 1.0mgL^{-1} 2,4-D and 1.0 mgL^{-1} BAP + 2.0 mgL^{-1} 2,4-D thus bringing the total combinations to 14. In addition, all media were supplemented with 30 gL^{-1} sucrose and adjusted to pH 5.8. Agar-agar (Merck; 0.7%) was used to solidify the media. For the purpose of micropropagation, baby food jars were used each containing 25 ml of the medium. For callus induction, 8 ml medium was dispensed into Pyrex culture vessels (25x 145 mm).

Maintenance of *In vitro* cultures: 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 first subculturing of shoots obtained from cormel was done after the removal of basal tuber. The calli initiated on different media were also maintained by subculturing in the same way.

Hardening and acclimatization: For the purpose of hardening and acclimatization to the field conditions, 10 plants (8-10 week-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 pre-sterilized 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 culture room. After further hardening of two weeks, the plants were shifted to the normal growth conditions of the field.

Culture environment: The cultures were kept in the growth room at 25 ± 2°C in 16 h photoperiod (2000-3000 lux).

Results and Discussion

a. Micropropagation: Shoot induction response of cormel explants was quite satisfactory (up to 90%) on both MS basal as well as MS half-strength media. Although shoot induction response from meristem explants was also obtained on both media, its rate was comparatively lower (up to 40%) as compared to the response by cormel explants. Hence, MS medium was supplemented with five different concentrations (0.5, 1.0, 1.5, 2.0 or 2.5 mgL^{-1}) of BAP for shoot induction from meristem explants. Shoot induction was possible using all the five media after 7 days of inoculation (data not shown) with a maximum rate of shoot induction (80%) at $1mgL^{-1}$ BAP. The results therefore, nearly corroborate the findings of Nagaraju & Parthasarathy (1995). They also used cormels of gladiolus as an explant source and found BAP ($0.75mgL^{-1}$) to be the most suitable growth regulator for multiple shoot induction.

We observed a linear relationship between the shoot induction and multiple shoot formation. Thus, in order to achieve multiple shoots from cormel and meristem explants, the same media combinations i.e., MS medium containing 0.5, 1.0, 1.5, 2.0 or 2.5 mgL^{-1} BAP were used. Initially, two to three shoot primordia were observed on the cultured explants depending on the media that multiplied as the explants were subcultured to the fresh media. These buds eventually gave rise to multiple shoots (Table 1). In our studies, both explants gave better multiplication response on MS medium containing $1mgL^{-1}$ BAP but the number of shoots obtained per culture vessel were more (16±0.95) when cormel was used as an explant as compared to those obtained from meristem explant (10±0.83). It must be mentioned here that the cultures derived from cormel explants on MS + BAP ($1mgL^{-1}$) kept on producing many more shoot primordia upon transfer to fresh medium. All what was required was to separate the longer shoots (2 cm or more) for rooting and transfer of remaining shoots (less than 2 cm) to the same fresh micropropagation medium. A total number of more than 50 shoots could thus be produced out of a single baby food jar over a period of 16 weeks which is quite an attractive gain compared to the contemporary literature. The potential for higher shoot number in other media over successive subcultures was comparatively low (data not shown).

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 concentration ($3.0mgL^{-1}$ and $4.0mgL^{-1}$ respectively for the two varieties). They attributed the higher dose requirement of BAP to be genotype-dependent. Although, the affectivity of BAP for shoot multiplication in gladiolus has already been reported, the nodal segments mostly remained the explant of choice. Grewel *et al*., (1995) used nodal segments of Gladiolus and got 14-20 shoot primordia within 4 weeks on MS medium containing $5mgL^{-1}$ BAP. Dantu & Bhojwani (1995) reported maximum number of shoots from axillary buds of Gladiolus on MS medium supplemented with $0.5mgL^{-1}$ BAP. Shoot multiplication of *Gladiolus grandiflorus* was also studied by Begum & Haddiuzaman (1995) who also used axillary buds as an explant and achieved maximum

shoot multiplication on MS medium containing 0.75mgL^{-1} BAP. From the literature available on *In vitro* manipulation in Gladiolus, it appears that the reports for multiple shoot formation from cormels are limited. Although it was difficult to quantify but we have observed that shoots obtained from cormel explants were generally healthier (better vigor) than those obtained from meristem explants. This clearly indicates that multiplication response also depends upon the nature of the explant used and cormels are a good source tissue for this purpose.

Rooting response for the shoots was 100% on half strength MS medium containing 2 mg^{L-1} IBA after 5 days of inoculation. Almost the same results (slightly less efficient but statistically non-significant) for rooting were obtained using half strength MS medium containing 0.5 mg^{L-1} NAA. The first medium was thus opted for routinely rooting *In vitro* shoots. Begum & Haddiuzaman (1995) also achieved the rooting of *In vitro* grown shoots using 0.5mgL^{-1} IBA in half strength MS basal medium. Thus rooting of multiple shoots at this high frequency in our studies supported acclimatization and hardening-off of micropropagated plants quite effectively. Up to 80% plants were successfully acclimatized in this study.

b. Callogenesis: We have found cormel explants to be a better callus source than the leaf explants both in quantitative as well as qualitative terms (data not shown). It is evident from Table 2 that callus initiation from slices of cormels was possible when NAA was used at a concentration of either 0.3 or 0.4mgL^{-1}. Moreover, callus initiation was also observed when BAP (1mgL^{-1}) was used in combination with 2mgL^{-1} 2, 4-D. Among the calli initiated on the above-mentioned two media using NAA alone, root organogenesis (rhizogenesis) was observed after one month of inoculation while shoot organogenesis occurred rarely. Boonvanno & Kanchanapoom (2000) induced callus from axillary buds of *Gladiolus* in the presence of 2.3μM NAA, on which rooting occurred without the formation of shoots. In this regard, the results from our studies also conform to the findings of Boonvanno & Kanchanapoom (2000). Root organogenesis in callus cultures was also observed in this study using MS media supplemented with either NAA concentration (0.3 or 0.3 mgL^{-1}). However, our results were not in harmony with Kamo (1995) who reported quite a high concentration of NAA (10 mgL^{-1}) for callus induction from cormel explants in *Gladiolus*. In addition, she reported callus induction from the same explants by using MS medium supplemented with either 0.5 mgL^{-1} 2, 4-D or 2.0 mgL^{-1} dicamba. In our studies, a relatively higher 2, 4-D level (2.0 mgL^{-1}) had to be supplemented with 1 mgL^{-1} BAP to obtain callus from cormel explants. On the other hand, success with relatively low NAA level (0.5 1mgL^{-1}) in our studies followed by a negative callus induction response by cormel explants at higher NAA levels (up to 4 mgL^{-1}) restricted us testing even higher NAA levels. Kumar *et al.,* (1999) used segments of cormels and inflorescence axes for callus induction and got maximum callogenic response of both explants on media containing 5.0μM BAP and 5.0 to 10.0μM 2, 4-D. Hence, we recommend using a lower 2, 4-D level (2 mgL^{-1}) supplemented with BAP (1.0 mgL^{-1}) in MS medium since it has shown promise in obtaining regenerable callus in our studies. Although we are not reporting here plant regeneration from callus cultures due to inconsistent results, yet a sporadic single shoot regeneration from callus cultures (up to 3rd subculture) obtained from cormel explants in MS medium supplemented with 2, 4-D (2 mgL^{-1}) and BAP (1.0 mgL^{-1}) have given us a direction towards achieving this objective.

Table 1. Effect of BAP on number of shoots from cormel and meristem explants of gladiolus at 16 hours photoperiod.

Medium composition	*Number of shoots (2 cm or more)	
	Cormel explant	**Meristem explant**
MS + 0.5mg^{L-1} BAP	11 ± 0.44	8 ± 0.32
MS + 1.0 mg^{L-1} BAP	16 ± 0.95	10 ± 0.83
MS + 1.5 mg^{L-1} BAP	12 ± 1.3	7 ± 0.79
MS + 2.0 mg^{L-1} BAP	10 ± 0.84	5 ± 1.4
MS + 2.5 mg^{L-1} BAP	6 ± 1.1	5 ± 0.46

*Comparison of number of shoots is based upon the data collected after 8 weeks of inoculation of both explants. Results are mean (± S. E) from ten replicate cultures

Table 2. Effect of different media on callogenic response and physical characteristics from cut cormel slices and leaf explants in gladiolus under 16-hours photoperiod conditions.

Medium composition (PGRs in mgL^{-1})	*Callus induction/physical characters	
	Cormels	**Leaf**
MS+ NAA (0.3)	Yellow, compact with smooth surface	-
MS + NAA (0.4)	Whitish, compact with rough surface	-
MS + NAA (0.5)	-	-
MS + NAA (1.0)	-	-
MS + NAA (2.0)	-	Greenish white, compact and rough
MS + NAA (3.0)	-	-
MS + NAA (4.0)	-	-
MS + NAA (0.4) + BAP (0.5)	-	-
MS + NAA (0.4) + BAP (1.0)	-	-
MS + NAA (0.4) + BAP (3.0)	-	-
MS + BAP (1.0) + 2,4- D (0.2)	-	-
MS + BAP (1.0) + 2,4-D (1.0)	-	-
MS+ BAP (1.0) + 2,4-D (2.0)	Whitish, compact with rough surface	-
MS+ 2,4-D (3.0)	-	Whitish, compact with smooth surface

-No callus induction

*Callus initiation in at least 50% (5 culture vessels per medium) of the two cultured explants where total number of cultures in each case was 10 thus representing a total of 280 culture vessels in the above experiment.

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