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Research Paper

Rapid Scale Micropropagation of Glycyrrhiza Glabra L. (Leguminoseae) A Valuable Medicinal Herb

Botany

KEYWORDS : Callus, Axillary bud, Organogenesis

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ABSTRACT

Method for in vitro micropropagation of Glycyrrhiza glabra L. by axillary bud proliferation was investigated by optimizing the concentrations and combinations of different phytohormone in MS medium. Nodal segment with axillary bud explants could be stimulated to form multiple shoots on MS medium augmented with BAP. The best proliferation (14.3 ±0.51shoots per plant) was achieved on BAP (5.0mgl^{-1}). Shoots could be easily rooted on MS medium containing 3 mgl^{-1} IAA. Plantlets were successfully acclimatized in green house and field conditions.

MATERIALS AND METHODS

Plant material

Cuttings of Glycyrrhiza glabra were procured from the department of Plant Breeding, Y.S. Parmar University of Horticulture and Forestry, Solan, H.P. The nodal segment with axillary buds (1-1.5cm in length) was subjected to preliminary washing under running tap water for 10-15min. to remove the microflora to a substantial extent. Explants were surface sterilized with 0.1% (w/v) Mercuric chloride and 7.5%Teepol for 10-12minutes. After repeated rinse with sterile distilled water, the nodal segment with axillary bud was cultured.

Culture media and culture Condition

Murashige and Skoog's (1962) medium with 3% Sucrose and 0.6 %(w/v) Agar-agar were used for successful establishment of Glycyrrhiza glabra. It was again supplemented with various plant growth regulators like 6-BAP, IAA, IBA and NAA in different concentrations and combinations. In all cases, the culture media were adjusted to pH 5.8 before autoclaving at 121°C, 1.05kg/cm^2 for 15 minutes. The cultures were maintained at 25 ± 1°C under 16 hours photoperiod of 3000lux intensity from cool white fluorescent tube light and at 55-60% constant relative humidity.

Organogenesis without callus intervention

For shoot bud initiation and rapid multiplication, the nodal segment with axillary bud explants **(fig.1)** were cultured separately on MS media supplemented with 6-BAP at the concentrations of (1mgl^{-1},2mgl^{-1},3mgl^{-1},4mgl^{-1},5mgl^{-1},6mgl^{-1}). These media were designated as Primary inductive media for axillary bud multiplication. Shoot buds thus formed after 3-4 weeks were divided into small groups (7-12 shoot buds in each group) and subculture on the fresh primary inductive medium for further multiplication and elongation. The subsequent sub cultures were also maintained keeping the duration of 4 weeks in each subculture.

Organogenesis through callus intervention

The leaf and nodal segments were used as explants for callus induction. These explants were inoculated in the agarified MS basal medium supplemented with 2, 4-D (1mgl^{-1}, 2.0mgl^{-1}and2.5mgl^{-1}). This was used for callus induction and proliferation. Callus thus formed was subculture at 30 days interval in the same medium.

For organogenetic regeneration, one month old calli were cultured on agarified MS medium with different concentrations and combinations of auxins (1mgl^{-1}of IAA, IBA and NAA) and cytokinin (1, 2.5 and 10mgl^{-1}of 6-BAP). Shoot bud thus formed after 4-5 weeks were divided into small groups (5-7 shoot buds in each group) and subculture on the fresh media of same composition for further multiplication and elongation was done only MS media. The subsequent subcultures were made after every 4 weeks.

Rooting of regenerated shootlets

Microshoots of 1.0-1.5cm length (3-4 week cultures) were excised and transferred to agar solidified MS media supplemented separately with IAA, IBA, NAA and liquid MS media without any growth regulator. After rooting, the complete plantlets were produced and grown in the full strength solid MS media without any growth regulator. These plants were then tried for hardening.

Transfer to the soil

One month old full grown plants of *Glycyrrhiza glabra* with well developed roots were transferred from the full solid MS media to the half strength liquid MS media and kept for 10-12 days. Then the plantlets were taken out, washed thoroughly and carefully with sterile water to remove all the remnants of agar media then transplanted in small pots confining sterile soilrite. The plants were kept exposed to the external environment for 2 hours and then taken to the glass house condition. The cycling of the environmental and glass house conditions was done for the well acclimatization of the plants with the external environment. The duration of exposure to the external environmental conditions was increased from 3 hours to 5 hours. After 15 days onwards the plants were kept in the normal environment for 6-8 hours. In all stage sufficient water was added to the soilrite in the pot. After complete 3 weeks the hardened plants were transferred to the larger pots containing soil and leaf compost.

Results

Multiplication of axillary buds

The multiplication of axillary buds began after 15-20 days in the MS media containing 6-BAP of different concentration like (1mgl^{-1}, 2mgl^{-1}, 3mgl^{-1}, 4mgl^{-1}, 5mgl^{-1},6mgl^{-1}and 7mgl^{-1}). The highest average number of shootlets developed per single axillary bud was 14.3 ±0.51 (Fig. 2, Table 1) was found in MS media containing 5 mgl^{-1} 6-BAP after 30-35 days of culture. In concentration of 3 mgl^{-1} 6-BAP and 4mgl^{-1} 6-BAP, the average number of shootlets was reduced to 3.0±0.25 (Table-1 Fig.5). The shoot lets were subculture on MS media without any growth regulators for elongation.

Organogenesis through callus phase

Callus was formed in callus inducing medium after 15-20 days of inoculation. In both leaf and nodal explants, MS media supplemented with 1.5 mgl^{-1} of 2, 4-D showed the best response. In the case of nodal segment as explants, callus was pale yellow in color and friable and in case of leaf explants, callus was light greenish and compact (Fig.3). In the present investigation the only leaf calli was showing the organogenic response. Shoot bud (greenish) began to appear from leaf calli after 21-25 days of culture in shoot bud inducing medium. For organogenetic regeneration, MS media supplemented with different concentration and combination of auxin and cytokinins were found be effective. Of the four auxins tested, except 2, 4-D, other three auxins with BAP induced shoot buds. Of all the combinations tried, 5 mgl^{-1} 6-BAP and 1.5 mgl^{-1} NAA showed the best re-

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sponse (Table-2 Fig.4).

Microshoots of 1.5cm to 2.5cm length (5-7 week old) were transferred to root inducing media. The agar solidified MS media without growth regulator was found to be the most effective for root induction. In the present material, 100% shoots formed roots after one month of culture. In case of IAA, primary roots were healthy with secondary roots. The best root initiation (68%) was (Fig.6) noted in MS media supplemented with 3 mgl^{-1} IAA (Table-3). The complete plant with well developed root system was mass propagated in the MS media without any growth regulator.

One month old plants with well developed roots were transferred from the culture tube to the pots containing sterile soilrite. At the time of transfer, the plantlets were attained the height of 5-7cm. after one month, successfully acclimatized tissue culture raised normal plants were transplanted to the normal environmental condition. (Fig.7-8)

Discussion

Shoot bud proliferation *in vitro* is usually considered a convenient route for micropropagation (Altman and Loberant, 1998; Bhojawani and Rhajadan, 1996; and Thorpe, 1994). Organogenesis starts with distinct organization of a group of new meristematic cells, directly within the explants. These were then transformed into a shoot or root meristem (Street, 1997; Bhojawani and Rhajadan, 1996; and Thorpe, 1994). The composition of nutrient medium, use of appropriate plant growth regulators and culture conditions are the important factors in the successful establishment of tissue culture.

To reduce the risks of somaclonal variability during the multiplication, apical and axillary meristems were the preferable explants for organogenesis (Geroge, 1993). In present investigation both kinds of regeneration of shoot buds from nodal segment with axillary bud and from calli were established.

In the present investigation, the role of cytokinin like 6-BAP in Organogenesis from nodal segment with axillary bud has been noted 5 mgl^{-1} concentration of BAP is found most effective in terms of number of shoot bud production in the present material. The promoting effect of cytokinins in shoot regeneration has also been established by different authors in Dendroacalamus hamiltonii (Chambers et al., 1991); Cajanas cajan (Shiva Prakash et al., 1994); Trifolium repense (White et al., 1994); Melia azedarach L. (Thakur et al., 1998); Ilex paraguariensis (Sansberro et al., 1999).

Reduction in the number of shoots were found in each node at higher concentrations of 6-BAP was also reported for several medicinal plants (Kukreja et al., 1990; Sen and Sharma, 1991; Hosoki and Katahira, 1994). The stimulatory effect of 6-BAP in bud break and multiple shoot formation has been reported for several medicinal and aromatic plant species including Adhatoda beddomei (Sudha and Seeni, 1994); Chlorophytum borivilianum (Purohit et al. , 1994; Ocimum sp. (Patnaik and Chand, 1998); Anacardium occidentale (Bogetti et al. , 1999).

In the present investigation, 2, 4-D is the most effective auxin for inducing callus tissue and for its maintenance. It was noted that friable and light green calli and friable and pale yellow calli were regenerating and nonregenerating in nature. The regeneration in the present material is dependent on the specific hormones and their balance in the medium. In the present investigation, cytokinin was apparently not essential for the induction of callus but it was essential for inducing the regeneration capacity in the callus tissue. In the present study, the regeneration of plants from callus tissue occurred when the callus obtained from the leaf segment and supplemented with combination of auxins (IAA, IBA and NAA,1mgl^{-1}) and cytokinin (6–BAP 1, 3, 5 and 10mgl^{-1}). NAA(1mgl^{-1}) and 6–BAP (5mgl^{-1}) showed best response for organogenesis via callus. Similar effect of auxin and cytokinin on organogenesis via calli was noted by several workers Mercier et al., 1992; George et al., 1993; Barna and Wakhlu, 1994; Ghosh and Sen, 1996; Zhang et al., 1997; Lin and Chang, 1998; George and Ravishanker, 1997; Tailang and Kharya, 1998; Sulaiman, 1994; Shivprakash et al., 1994; Miyoshi and Asakura, 1996; Sudha and Seeni, 1994; Chand et al., 1997; Anand and Hariharn, 1997. Thus it may be stated physiological and cellular state of the donor plant is an important factor in organogenetic expression of the callus tissue.

In the present material, best rooting of the regenerated shotlets was found in MS media without any growth regulator. Besides this media, MS media with different auxins (IAA, NAA and IBA) as supplements also promote the development of roots in the regenerated shoots. The similar finding was noted in Solanum rickii (Kochevenko et al., 1996). In present study, about 86% shoots rooted on hormone-free medium i.e. MS based medium only. Roots formed on NAA containing medium were hypertrophied. IBA induced roots were brittle. But IAA formed normal roots. In terms of rooting efficiency IAA at a concentration of 3 $mgl^{-1}$was found to be most effective. Similar good response of IAA in root initiation was noted in a number of plant species such as Santalum album (Lakshmi sita et al., 1979); Vigna radiata (Gulati and Jaiwal, 1994); Vitex negundo L. (Sahoo and Chand, 1998); Azadiractha indica (Eeswara et al.,1998).

Conclusion

From the foregoing it may thus be concluded that for rapid micropropagation of Glycyrrhiza glabra L., 5mgl-1BAP is best effective. MS medium with 3mgl-1 IAA is the best for promoting root differentiation for shoots. Plantlets thus obtained can be successfully acclimatized in both green house and field conditions for reaping the benefits of its medicinal virtues.

Table-1: Formation of shootlets from the axillary bud of *G. glabra* in MS media with different concentrations of 6-BAP

Concentrations (mgl^{-1}) 6-BAP	Number of shootlets	Range of shoot Length (cm)
1.0	2.2±0.33	1.5±0.09
2.0	3.2±0.13	3.0±0.13
3.0	7.4±.30	4.5±0.26
4.0	9.2±0.74	3.5±0.11
5.0	14.3±0.51	2.0±0.04
6.0	3.0±0.25	1.5±0.08
7.0	0.0	-

***Data presented as mean value ± standard error of shootlet number formed from single axillary bud after 35days of culture.**

Table-2: Shoot regeneration from callus of *G.glabra* under the interaction of auxin and cytokinin

Treatment Concentration(mgl^{-1})	*Shoot formation	Survival Rate (%)	*Shoot length (cm)
IAA + 6-BAP	0	Nil	-
1 mgl^{-1} +1 mgl^{-1}	1.5±0.07	80%	1.5±0.09
1 mgl^{-1} +2 mgl^{-1}	6.7±0.14	100%	3.7±0.09
1 mgl^{-1} +5 mgl^{-1}	0	Nil	-
1 mgl^{-1} + 10 mgl^{-1}			
NAA + 6-BAP	1.7±0.28	70%	1.2±0.07
1 mgl^{-1} +1 mgl^{-1}	5.7±0.3	85%	2.2±.31
1 mgl^{-1} +2 mgl^{-1}	10.3±0.47	85%	2.0±0.17
1 mgl^{-1} +5 mgl^{-1}	0	Nil	-
1 mgl^{-1} + 10 mgl^{-1}			
IBA + 6-BAP	2.5±0.37	70%	2.2±0.31
1 mgl^{-1} +1 mgl^{-1}	6.2±0.79	100%	2.7±0.16
1 mgl^{-1} +2 mgl^{-1}	3.1±0.5	60%	2.0±0.17
1 mgl^{-1} +5 mgl^{-1}	0	Nil	
1 mgl^{-1} + 10 mgl^{-1}			

***Data presented as mean value ± standard error of shootlet number formed per gm. of callus after 35-40 days of culture.**

Table–3: Response of shoots in different rooting media

Media used	Concentration (mgl^{-1})	*Number of shoots with roots
Liquid MS (devoid of Growth regulator)	-	86
MS(Solid)+IAA	1 3 5	50 68 35
MS(Solid)+IBA	1 3 5	23 49 35
MS(Solid)+NAA	1 3 5	07 0 0

***Data represented as the % of shoots that formed roots after 30 days of culture.**

Fig. 1

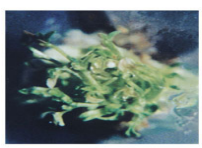

Fig. 2

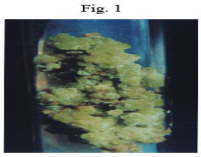

Fig. 3

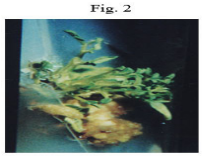

Fig. 4

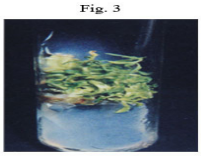

Fig. 5

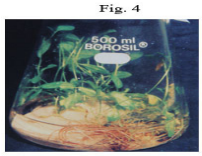

Fig. 6

Fig. 7

Fig. 8

Fig.1 Multiplication of axillary buds in *G. glabra* with the explant nodal segment.
Fig. 2 Axillary bud multipliation in MS media supplemented with 5mg/l 6-BAP after 35 days.
Fig. 3 Induction and proliferation of calli in *G. glabra* 1.5mg/l 2,4-D after 25 days.
Fig. 4 Regeneration of shootbuds from leaf calli in MS media containing 5mg/l 6-BAP and 1mg/l NAA.
Fig. 5 Reduction in Shootlets in concentration of 3 and 4mg/l 6-BAP.
Fig. 6 Rooting of the regenerated shootlets of *G. glabra* in liquid MS media devoid of growth regulators after 30 days.
Fig. 7 Hardening of the regenerated plats of *G. glabra*.
Fig. 8 One and half month old hardened plant in pots containing soil and leaf compost.

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