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Effect of Plant Growth Regulators on *in vitro* Morphogenic Response of Gliricidia [*Gliricidia sepium* (Jacq.) Steud.]

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ABSTRACT

Background: The present investigation was carried out with the objective of optimization of rapid micropropagation protocol and to evaluate effect of different concentrations of plant growth regulators added singly or in combinations on *in vitro* culture of *Gliricidia sepium*.

Method: Explants (Soft nodal stem segments) were inoculated on MS medium containing varying concentrations of cytokinins and auxins either singly or in combinations. The cultures were incubated at 25±2°C for 14: 10 hour's photoperiod with a light intensity of 3000-3500 lux.

Result: Maximum number of shoot bud induction obtained when MS medium was supplemented with 0.5 mg/l BAP with 100 per cent frequency. Highest frequency (100%) of rooting was observed at 0.5 mg/l of IAA. 2.0 mg/l NAA showed highest number of roots/ explant but the roots were thick. For shoot bud induction, BAP was found to be most effective among all the plant growth regulators. For root induction, both NAA and IAA were found to be effective but IAA exhibited higher frequency of rooting as compared to NAA.

Key words: Auxins, Cytokinins, *Gliricidia sepium,* Micropropagation, MS medium.

INTRODUCTION

Gliricidia [*Gliricidia sepium* (Jacq.) Steud.] is a medium size, semi deciduous multipurpose forage tree belongs to family *Fabaceae*. Central America and possibly South America are believed to be the native place of this forage tree (Hughes, 1987 and Lavin and Sousa, 1995). It is distributed over Tropical America, Africa and Fiji. In India, it is mainly cultivated in Tamil Nadu, Andhra Pradesh, Maharashtra and Karnataka. It has diploid chromosome number (2n=22). The genus *Gliricidia* has three main species namely *Gliricidia sepium, Gliricidia maculata* and *Gliricidia brenningii* (Hughes, 1987 and Lavin *et al*. 1991). It has hermaphrodite flowers. Gametophytic system of self incompatibility mechanism is operational in case of *Gliricidia sepium*, whereby self pollen can germinate but is prevented from fertilizing the ovule (Stewart *et al*. 1996).

This forage tree is used as fodder, fuelwood, poles, live fencing, erosion control, support for other crops, and soil nutrient enhancer through biological nitrogen fixation and has vigorous response to a wide range of management techniques. It is most widely used and known for its high nutritive value for ruminants, high leaf biomass production, sustainable over many years of repeated pruning or croppicing and provision of dry season green fodder.

Micropropagation technology offers many advantages when compared with other conventional propagation techniques. In micropropagation, product development is by rapid increase in number of propagules both in asexually propagated and sexually increased (*via* seed) materials. The resulting product can have high degree of uniformity and large number derived from desired genotype. This crop has tremendous scope in Rajasthan for forage and green manure. However, seed setting is a major problem in the arid condition along with germination of seed. It hinders its propagation at a large scale. Considering these problems and limitations, present study has been undertaken to explore potential of *in vitro* multiplication for large scale propagation and to evaluate effect of different concentrations of plant growth regulators added singly or in combinations on *in vitro* culture of gliricidia.

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MATERIALS AND METHODS

The present investigation was carried out during year 2015-2016 at the Department of Plant Breeding and Genetics, S. K.N. College of Agriculture, SKNAU, Jobner.

Plant material and Sterilization

Soft nodal stem segments were used as explants obtained from herbal garden maintained under Department of Plant Breeding and Genetics, S.K.N. College of Agriculture, Jobner. Explants were washed thoroughly in running tap water. These were again washed with liquid detergent

(Rankleen) with vigorous shaking. After washing with detergent, explants were again washed with running tap water to remove any trace of detergent. Explants were surface sterilized with 0.1 per cent $HgCl_2$ in a laminar air flow cabinet for 3 minutes. Aseptic condition of transfer area is maintained by installing an HEPA filter ventilation unit. Laminar airflow hoods are sterilized by wiping the working surface with 70 per cent ethyl alcohol and by switching on UV light for 30 minutes.

Media preparation and culture conditions

All chemicals used in the present study were of analytical grade. Murashige and Skoog (1962) medium was used throughout the course of investigation. A beaker was filled with 800 ml of double distilled water. 4.4 g of MS medium (powdered), 30 g of sucrose, and 8 g of agar were added to the beaker and heated to mix well. Plant growth regulators were added to the basal medium at the end before autoclaving. The pH of the medium was adjusted at 5.84 using 1N NaOH or 1N HCl solutions. Volume was made up to 1 litre. Culturing flask and test tubes were filled with media. The culture media contained in a conical flask and test tubes sealed with cotton plugs and covered with aluminium foils were autoclaved at 15 psi and 121°C for 30 minutes. Exposure time depends on the volume of the liquid to be sterilized. After autoclaving, media were stored in the dark for 48 hours at 25 + 2°C.

Different concentrations of plant growth regulators were incorporated singly and in combinations in the MS medium for shooting and rooting are as follows: Plant growth regulators incorporated singly in the medium. (a) Cytokinins (BAP/Kn) : 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 and 2.5 mg/l. (b)Auxins(IAA/NAA): 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 and 2.5 mg/l. Plant growth regulators incorporated in combinations. (a) BAP (0.5 and 2.0 mg/l) + NAA/IAA (0.5 and 2.0 mg/l). (b) Kn (0.5 and 2.0 mg/l) + NAA/IAA (0.5 and 2.0 mg/l). For rooting of *in vitro* induced shoots, shoots of 1.0-1.5 cm length were harvested from *in vitro* proliferated multiple shoots and subjected to different levels of auxins (IAA/NAA) for the induction of roots. (a) IAA - 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 and 2.5 mg/l. (b) NAA - 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 and 2.5 mg/l.

Inoculation of explants

After sterilization, the explants were inoculated on culture media aseptically. For inoculation, explants were transferred to a large sterile glass petriplates with the help of sterile forceps under strict aseptic conditions. Here, the explants were further trimmed to desired size upto 2-3 cm with sterile scalpel blade. After cutting explants of suitable size, these were transferred to culture test tubes and borosil flasks containing MS medium supplemented with different plant growth regulators. After vertically inoculating the explants in the culture test tubes and borosil flasks, mouth of the test tubes and borosil flasks were quickly flamed and were closed with non adsorbent cotton plug. All cultures were incubated at 25+2°C under fluorescent light for 14: 10 hour's photoperiod.

Hardening

After 30-40 days of culture on rooting media, the plantlets were shifted to the root trainer for their hardening. After removing solidifying agar media by dipping in lukewarm water, plantlets were dipped in 1 per cent w/v solution of bavistin to prevent any fungal infection. Plantlets were carefully planted in root trainers containing 1:1 mixture of sterile soil and vermiculite and watered at an interval of 2-3 days to maintain moisture in the root trainers for 10 days in the culture room. Then the plants were shifted to the shade house with indirect sunlight.

Data recording and analysis

Each treatment combination was replicated 10 times. Cultures were observed periodically and following observations were recorded: percentage of explants producing shoot, number of shoots/explant, Percentage of explants producing root, number of roots/explants. Data were analyzed for mean and standard error.

RESULTS AND DISCUSSION

Effect of cytokinins added singly (BAP and Kinetin)

When explants were inoculated on MS medium supplemented with various concentration of cytokinins (BAP and Kinetin), soft nodal stem segments induced variable response. Predominantly shoot induction was observed within 8-16 days of incubation. When MS medium supplemented with BAP singly (0.1-2.5 mg/l), it induced shoot buds at all the concentrations. Maximum number of shoot bud (3.1) was observed at 0.5 mg/l with 100 percent frequency (Fig 1a) followed by 0.6 (2.4) mg/l and 0.4 (2.3) mg/l with 60 and 100 per cent frequency, whereas minimum number of shoot bud (1.1) was observed at 2.0 mg/l BAP with 60 per cent frequency followed by 2.5 (1.3) mg/l and 0.7 (1.3) mg/l with 40 and 60 per cent frequency. In BAP supplemented medium, proliferation of shoot buds were higher as compared to the other plant growth regulators when supplemented in the basal medium singly (Table 1). Replacement of BAP with Kinetin in the MS medium induced shoot buds at most of the concentration except 0.1 mg/l. But frequency of shoot bud induction was lower as compared to BAP. Maximum shoot buds (2.4) were observed at 0.8 mg/l Kinetin with 80 per cent frequency followed by 0.6 (2.2) mg/l and 0.7 (2.1) mg/l with 60 per cent frequency. Minimum number of shoot bud (1.1) induction was observed at 0.2 mg/l Kinetin with 40 per cent frequency.

The most striking influence on bud breaks and shoot multiplication has been found with cytokinins (Normanly *et al*. 1995). BAP and Kinetin are the most commonly used cytokinins for micropropagation. In the current investigation, high shoot buds were observed when soft nodal stem segments were inoculated on the basal medium containing 0.5 mg/l BAP. This result was in accordance with results

obtained by Kumari (2012) with respect to plant growth regulator (BAP). She observed maximum rate of shoot multiplication at 5.0 mg/l BAP in *Bauhinia variegate*. However, in the present study maximum shoot proliferation was observed at 0.5 mg/l BAP. This variation might be due to differences in genera. Role of BAP for shoot induction was also observed by Chauhan and Jha (2018) in *Accasia mangium*, Borthakur *et al*. (2012) in *Albizzia chinensis*, Ismail *et al*. (2012) in *Acacia auriculiformis* and Kumar and Singh (2010) in *Prosopis cineraria*. These results were in close agreement with present study. However, range of BAP concentration was different which might be due to different genera.

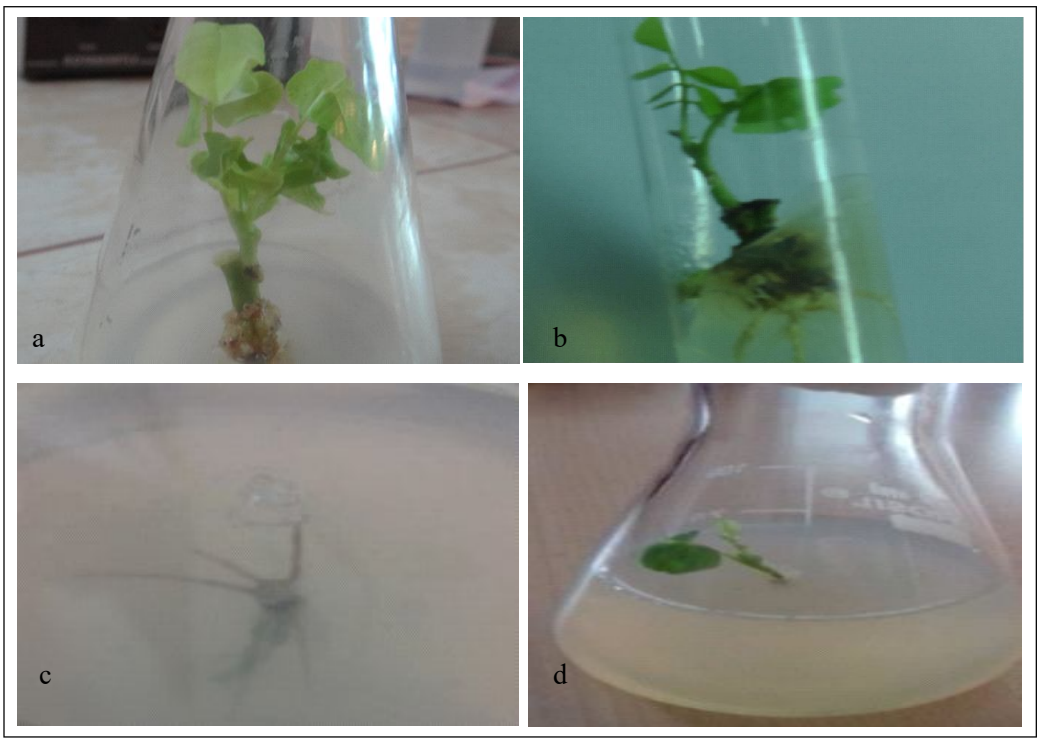

Fig 1: *In vitro* propagation of *Gliricidia sepium* (a) Shoot induction in *Gliricidia sepium* on MS medium supplemented with 0.5 mg/l BAP. (b) Root induction in *Gliricidia sepium* on MS medium supplemented with 0.5 mg/l IAA. (c) Root induction in *in vitro* proliferated shoot in *Gliricidia sepium*. (d) Complete *in vitro* generated plantlet.

Table 1: Morphogenetic effect of cytokinins (BAP and Kinetin) added singly in the MS medium in *Gliricidia sepium*.

Concentration mg/l	BAP		Kinetin	
	No. of shoot buds/explants Mean±SE n=10	Frequency (%)	No. of shoot buds/explants Mean±SE n=10	Frequency (%)
0.1	2.2±0.20	80	-	-
0.2	2.1±0.17	80	1.1±0.10	40
0.3	2.0±0.15	80	2.1±0.18	40
0.4	2.3±0.19	100	1.4±0.49	40
0.5	3.1±0.18	100	1.3±0.15	40
0.6	2.4±0.16	60	2.2±0.20	60
0.7	1.3±0.16	60	2.1±0.18	60
0.8	2.0±0.15	60	2.4±0.16	80
0.9	2.1±0.18	60	1.5±0.17	60
1.0	1.4±0.49	60	1.3±0.15	60
1.5	2.2±0.20	60	1.5±0.16	60
2.0	1.1±0.10	60	1.6±0.49	60
2.5	1.3±0.15	40	1.6±0.49	40

(-)= No morphogenetic effect.

Effect of auxins added singly (IAA and NAA)

When MS medium was supplemented with various auxins (IAA and NAA), soft nodal stem segment induced both shoot and root. However, the frequency of shoot morphogenetic response was less in comparison to cytokinins. When explants were inoculated on MS medium supplemented with various levels of IAA (0.1-2.5 mg/l), a very slow response of shoot bud multiplication was observed. Shoot bud induction was observed at 0.5-0.6 mg/l concentrations with 20 per cent frequency. Maximum shoot buds (1.4) were observed at 0.6 mg/l IAA while minimum at 0.5 mg/l IAA (Table 2). Both lower and higher levels of IAA were insufficient to induce shoot buds in the explants (Table 2). Root induction frequency ranged from 20-100 per cent. Thin and long roots (4.1) were observed in the medium supplemented with 0.5 mg/l IAA with 100 per cent frequency (Fig 1b). Increase in IAA concentration beyond 0.8 mg/l resulted in reduced root induction frequency.

Root induction frequency was more in IAA supplemented medium as compared to NAA supplemented medium (Table 2). There was no any sign of shoot induction at all the levels of NAA. Thick and small roots were observed at all the levels of NAA (0.1-2.5 mg/l). Root induction frequency ranged from 20-80 percent. Maximum roots were observed at 2.0 mg/l with 30 percent frequency (Table 2), roots were thick and hairy. Maximum root induction frequency (80 per cent) was exhibited at 0.2 mg/l NAA. Number of root induction was more in NAA supplemented medium as compared to IAA supplemented medium.

Auxins are mostly used for rooting and callus induction. Rooting response of shoot was reported to be controlled by growth regulators in the medium (Bhojwani and Razdan, 1992) and basal salt composition (Zimmerman and Broome, 1981). For most of the species, auxin is required to induce root. NAA and IBA are most commonly used for root induction (Bhojwani and Razdan, 1992). By the use of the IBA in many plants such as *Lycopersicon esculentum* (Sibi, 1982), *Hydichium roxburgii* (Tripathi and Bitallion, 1995), Carnation (Werker and Leshem, 1987) *in vitro* rooting was obtained. Borthakur *et al.* (2012) reported highest rooting frequency in *in vitro* grown shoots of *Albizia chinensis* on MS medium supplemented with IAA with an average of 2 roots/explant which was similar to the present investigation where high rooting frequency was observed at (0.5 mg/l) IAA in *Gliricidia sepium*. Variation in concentration in both the study may be due to the differences in the genera. Abhijeeta *et al.* (2018) and Jain and Chaturvedi (2004) also observed higher frequency of rooting in pigeonpea, when MS medium was supplemented with IAA.Almeida and da Silva (2021) observed higher percentage of rooting when MS medium was supplemented NAA.

Effect of cytokinins (BAA/Kinetin) and auxins (IAA/NAA) added in combination

Only few combinations induced shoot buds in explants. However, root induction was observed at all the combinations. Interaction of BAP with IAA showed better results as compared to the other combinations such as BAP + NAA, Kinetin + IAA and Kinetin + NAA for shoot bud induction. Maximum number of shoot bud (2.3) was observed at 0.5 mg/l BAP + 0.5 mg/l IAA with 60 per cent frequency (Table 3). There was no shoot bud induction at other combinations of BAP and IAA. Combination of BAP with IAA also showed root induction at all the levels. However, number of roots and frequency of root induction was lower as compared to IAA supplemented in the basal medium alone. Maximum number of root was observed at 0.5 mg/l BAP + 0.5 mg/l IAA with 60 per cent frequency

Table 2: Morphogenetic effect of auxins (IAA and NAA) added singly in the MS medium in *Gliricidia sepium*.

	IAA				NAA			
Concen--tration mg/l	No. of shoot buds/explants Mean±SE n=10	Frequency (%)	No. of roots/ explants Mean±SE n=10	Frequency (%)	No. of shoot buds/explant Mean±SE n=10	Frequency (%)	No of roots /explant Mean±SE n=10	Frequency (%)
0.1	-	-	2.4±0.16	40	-	-	4.4±0.16	60
0.2	-	-	3.1±0.18	60	-	-	5.10±0.18	80
0.3	-	-	2.4±0.22	20	-	-	6.4±0.31	60
0.4	-	-	3.4±0.22	60	-	-	5.4±0.22	60
0.5	1.1±0.10	20	4.1±0.17	100	-	-	6.1±0.23	60
0.6	1.4±0.49	20	3.2±0.24	100	-	-	4.2±0.13	60
0.7	-	-	2.1±0.17	60	-	-	5.1±0.23	60
0.8	-	-	2.4±0.16	60	-	-	6.4±0.31	60
0.9	-	-	2.5±0.21	60	-	-	6.5±0.27	60
1.0	-	-	2.3±0.15	20	-	-	7.3±0.12	20
1.5	-	-	3.5±0.16	20	-	-	6.2±0.19	20
2.0	-	-	3.6±0.16	20	-	-	10.1±0.17	30
2.5	-	-	2.5±0.26	20	-	-	8.1±0.18	30

(-)= No morphogenetic effect.

(Table 3). Lower level of BAP with both higher and lower level of IAA induced more roots in explants as compared to high level of BAP with IAA. Combination of BAP (0.5 mg/l) with (2 mg/l) NAA in basal medium induced shoot buds with 40 per cent frequency. Rest of the combinations did not proliferate shoot buds. Interaction of BAP with NAA did not show significant result at all the levels except 0.5 mg/l BAP + 2.0 mg/l NAA mg/l. Addition of BAP with NAA to the basal medium resulted in better frequency of rooting as compared to other combinations. Maximum number of root was obtained at 0.5 mg/l BAP + 2.0 mg/l NAA (Table 3). When combinations of Kinetin + IAA and Kinetin + NAA were used in basal medium proliferated limited number of shoot buds. Combination of Kinetin with IAA and NAA showed lower root induction frequency in comparison to combination of BAP with IAA and NAA. Maximum number of roots (4.2) was observed at 0.5 mg/l Kinetin with 2.0 mg/l NAA (Table 4). There is numerous synergistic effects of cytokinins and auxins on shoot bud multiplication and requirement for growth regulators varies with the crop and the mode of multiplication.

Root induction

Three to four centimeters long shoots were excised individually from the proliferated shoot clumps and cultured on rooting medium. In present investigation, root induction was assessed in the media supplemented with different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 and 2.5 mg/l) of auxins (IAA and NAA). Majority of *in vitro* shoots developed roots within 15 - 20 days of incubation. Thin and long roots were observed at all the levels of IAA (0.1-2.5 mg/l) (Fig 1c.). Root induction frequency was more in IAA supplemented medium as compared to NAA supplemented medium. Thick and small roots were observed at all the levels of NAA (0.1-2.5 mg/l). Best result was obtained at 0.5 mg/l IAA.

Hardening of plantlets

After 30-40 days of culture of proliferated shoots on rooting medium (MS medium supplemented with 0.5 mg/l IAA), complete plantlets were obtained (Fig 1d.). These plantlets were shifted to root trainer for their hardening prior to final transfer to the soil in natural condition.

Table 3: Morphogenetic effect of cytokinin (BAP) and auxins (IAA/NAA) added in combination in the MS medium in *Gliricidia sepium.*

Concentration mg/l	Morphogenic effect			
	No of shoot/explants Mean±SE n=10	Frequency (%)	No of root/explants Mean±SE n=10	Frequency (%)
BAP+IAA				
0.5+0.5	2.3±0.19	60	3.1±0.18	60
0.5+2.0	1.2±0.13	40	2.4±0.22	40
2.0+0.5	-	-	2.1±0.17	20
2.0+2.0	-	-	2.3±0.15	
BAP+NAA				
0.5+0.5	-	-	3.4±0.22	40
0.5+2.0	2.2±0.20	40	4.4±0.16	60
2.0+0.5	-		4.2±0.13	60
2.0+2.0	-		3.6±0.16	60

(-)= No morphogenetic effect.

Table 4: Morphgenic effect of cytokinin(Kinetin) and auxins (IAA/NAA) added in combination in the MS in *Gliricidia sepium.*

Concentration (mg/l)	Morphogenic effect			
	No of shoot/explants Mean±SE n=10	Frequency (%)	No of roots/explants Mean±SE n=10	Frequency (%)
Kinetin + IAA				
0.5+0.5	1.1±0.10	20	3.2±0.24	40
0.5+2.0	-	-	2.1±0.17	20
2.0+0.5	-	-	2.3±0.15	20
2.0+2.0	-	-	2.1±0.17	20
Kinetin + NAA				
0.5+0.5	-	-	3.4±0.22	40
0.5+2.0	1.3±0.15	40	4.2±0.16	40
2.0+0.5	-	-	3.6±0.13	40
2.0+2.0	-	-	4.1±0.17	60

(-)= No morphogenetic effect.

CONCLUSION

From above mentioned experiment, it may be concluded that optimum concentration of BAP for buds proliferation of this forage tree (*Gliricidia sepium*) was 0.5 mg/l. Better rooting frequency was observed on the media supplemented with 0.5 mg/l IAA. In the medium supplemented with various concentration of IAA, *Gliricidia sepium* produced thin and long roots with higher frequency. NAA was also found effective for rooting but NAA supplemented media produced thick and short roots. On the basis of the present investigation, it is recommended that the above given protocol may be used by other researchers for further validation and mass multiplication of *Gliricidia sepium* under *in vitro* condition.

Conflict of interest: None.

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