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Effects of Antioxidants in Controlling Phenolic Exudates in *in vitro* Culture of Gliricidia [*Gliricidia sepium* (Jacq.) Steud.]

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A B S T R A C T

The present investigation was carried out to study the influence of antioxidants on *in vitro* culture of *Gliricidia sepium.* Soft nodal stem segments were inoculated on MS medium supplemented with desired concentrations plant growth regulator such as cytokinins (BAP/Kinetin) and different antioxidants. Antioxidants viz., activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the culture medium and enhance the rate of micropropagation. All cultures were incubated at 25+2^{0}C under fluorescent light in a 14: 10 hour's photoperiod. Maximum number of shoot bud induction was obtained when MS medium is supplemented with 0.5 mg/l and 250 mg/l ascorbic acid.

Introduction

Gliricidia sepium is a medium-sized leguminous tree.It belongs to family *Fabaceae*. It has diploid number chromosomes 2n=22. Central America and possibly South America are believed to be native place of this forage tree (Hughes, 1987 and Lavin and Sousa, 1995). It is distributed over Tropical America, Africa and Fiji. It is an introduced forage tree in India. In India, it is mainly cultivated in Tamil Nadu, Andhra Pradesh, Maharashtra and Karnataka.

Gliricidia sepium can perform well on marginally saline vertisols. There is a hermaphrodite flowering system coupled with obligate outcrossing and a strong self-incompatibility mechanism (WAC, 2005). *G. sepium* is an extremely versatile nitrogen-fixing agroforestry tree that can be incorporated in diverse ways into many different smallholder farming systems and provide a range of wood and leaf products including fuelwood, construction poles, crop supports, green manure, fodder and bee forage (Simons and Stewart, 1994; Stewart, 1996). In many areas seed setting is extremely low and natural regeneration is poor. 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. Micropropagation technology offers many advantages when compared with other more conventional propagation methods. It was noticed that plant phenolic increased the rigidity of plant cell wall and acted as a molecular bridges between cell wall components (Ozyigit, 2008). During micropropagation, the exudation is very common and it often influences the results. Phenolic secretions and other exudates in plant tissue culture systems lessen explant initiation, growth, and development (Kerns *et al.,* 1986). The antioxidant has been successfully used in past to inhibit the exudation of phenols and reduced oxidative browning in various plant species (Abdelwahd *et al.,* 2008)

Materials and Methods

In order to observe the effect of different antioxidant, present research was conducted. Soft nodal stem segments of *Gliricidia sepium* were used as explant. Explants were washed with detergent and rinsed with running tap water to remove dirt. Soft stem nodal segments were cut into 1.5cm to 2.0cm length with single node. In laminar airflow chamber, explants were sterilized with 0.1 percent mercuric chloride and finally used for raising *in vitro* cultures.

Murashige and Skoog (1962) medium was used throughout the course of investigation. MS medium were supplemented with desired concentration plant growth regulator such as cytokinins (BAP/Kinetin) and different antioxidants. Antioxidants viz: activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the culture medium and enhance the rate of micropropagation. Different concentrations of various antioxidant were tested at most responsive level of plant growth regulators where maximum shoot development was observed. Following levels of antioxidants were used:

Activated charcoal (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/l).

Ascorbic acid (50,100,150, 200, 250 and 300 mg/l).

Citric acid (10, 20, 30, 40, 50 and 60 mg/l).

Polyvinylpyrrolidone (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l).

The pH of the culture medium was adjusted at 5.84 using 1N NaOH or 1N HCl solution before autoclaving. The culture media were autoclaved at 15 psi and 121 ^{0}C for 15- 40 minutes. After sterilization the explants were inoculated on culture media aseptically. For inoculation, explants were transferred to large sterile glass petriplates with the help of sterile forceps under strict aseptic conditions. Explants of suitable size were transferred to culture test tubes, phyta jars and borosil flasks containing MS medium supplemented with different plant growth regulator and antioxidants. All cultures were incubated at 25+2^{0}C under fluorescent light in a 14: 10 hour's photoperiod.

Each treatment combination was replicated 10 times and whole experiment was repeated twice to obtain unbiased results. 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/explant and Number of days taken to for development of shoots and roots. The experiment was conducted in completely randomized design and data were analyzed for mean and standard error.

Results and Discussion

The most striking effect of bud break and shoot multiplication have been found with many antioxidant (Wang *et al.,* 2002 and Ujjwala, 2006). Activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone are most commonly used antioxidants for micropropagation.

In present study, when different antioxidants incorporated singly in MS medium supplemented with responsive level (0.5 mg/l BAP) of plant growth regulator for shoot multiplication elicited different response for shoot bud induction because it controls the accumulation of inhibitory substance (phenolic compounds) in the growth medium.

Effect of activated charcoal

When activated charcoal is added in the basal MS medium with micropropagation protocol (0.5 mg/l BAP), it induced shoots at all the levels (0.5– 3.0 g/l). Maximum number of shoot bud (5.0) was observed at 2.0 g/l with 100 per cent frequency (Table 1). The result found in case of activated charcoal was in close agreement with effect of antioxidant on micropropagation in other crops (Wu and Xi, 2002) where activated charcoal was prime antioxidant used for successful shoot multiplication. In other studies, North *et al.,* (2012) recorded 53% reduction of phenols in media supplemented with activated charcoal in *Strelitziareginae.*

Fig.1 Shoot induction in *Gliricidia sepium* on MS medium supplemented with 0.5 mg/l BAP and 250 mg/l ascorbic acid

Table.1 Effect of different antioxidants supplemented in the MS medium along with 0.5 mg/l BAP on shoot bud induction in *Gliricidia sepium*

Morphogenetic response of soft nodal stem segment on different antioxidants											
Activated charcoal			**Ascorbic acid**			**Citric acid**			**Polyvinylpyrollidone**		
Concen-tration (g/l)	**No of shoot buds /explant Mean ± SE n =10**	**Frequency (%)**	**Concentration (mg/l)**	**No of shoot bud /explant Mean ± SE n =10**	**Frequency (%)**	**Concentration (mg/l)**	**No of shoot buds /explant Mean ± SE n =10**	**Frequency (%)**	**Concentration (mg/l)**	**No of shoot buds /explant Mean ± SE n =10**	**Frequency (%)**
0.5	3.1 ± 0.21	80	50	3.2 ± 0.24	60	10	3.8 ± 0.13	80	0.5	2.4 ± 0.16	60
1.0	3.2 ± 0.24	80	100	3.6 ± 0.16	60	20	3.2 ± 0.20	80	1.0	2.6 ± o.26	60
1.5	4.1 ± 0.17	80	150	3.8 ± 0.13	60	30	3.0 ± 0.21	60	1.5	3.1 ± 0.10	60
2.0	5.0 ± 0.34	100	200	4.0 ± 0.21	80	40	2.4 ± 0.22	60	2.0	3.5 ± 0.16	80
2.5	3.3 ± 0.21	80	250	5.1 ± 0.17	100	50	2.2 ± 0.13	60	2.5	3.4 ± 0.22	60
3.0	4.2 ± 0.2	80	300	4.6 ± 0.22	80	60	2.3 ± 0.15	40	3.0	2.8 ± 0.13	60

Effect of ascorbic acid

When ascorbic acid is added in the MS medium with micropropagation protocol (0.5 mg/l BAP), it induced shoot buds at all the levels (50 - 300 mg/l). Maximum number of shoot bud induction (5.1) was observed at 250 mg/l ascorbic acid level with 100per cent frequency (Fig. 1). Minimum number of shoot bud (3.2) was obtained 50 mg/l with 60 per cent shoot bud induction frequency (Table 1). Ndakidemi *et al.,* (2014) established that the best result for controlling lethal browning was obtained when *B. huillensis* nodal segments were cultured on medium supplemented with 5 µM BAP and incorporated with 200 - 250 mg/litre of ascorbic acid which is in close agreement with the present investigation.

Effect of citric acid

Supplementation of citric acid in the basal MS medium containing 0.5 mg/l BAP induced shoots at all the levels (0.5- 3.0 mg/l). Maximum shoot bud (3.8) was observed at 10 mg/l citric acid with 80 per cent shoot bud induction frequency. Increasing level of citric acid in medium showed decrease in number of shoot multiplication. The frequency of shoot multiplication ranged from 40 – 80 per cent (Table 1).

Effect of polyvinylpyrrolidone

Addition of polyvinylpyrrolidone in culture vessels with responsive level of plant growth regulator (0.5 mg/l BAP) did not show very good results on shoot multiplication in explants. It induced shoots at all the levels (0.5- 3.0 mg/l). Maximum shoot bud (3.0) was observed at 3.0 mg/l with 80 per cent shoot bud induction frequency (Table 1).

In conclusion, it was found that addition of ascorbic acid (250 mg/l) in the MS medium with 0.5 mg/l BAP appeared most optimum level of antioxidant for maximum shootbud proliferation and in controlling harmful effect of phenolic compounds in culture medium with 100 per cent frequency followed by 2.0 g/l level of activated charcoal. Polyvinylpyrrolidone was found to be least effective in controlling harmful effect of phenolic compounds.

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