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Research Article

Seasonal variation in contamination and browning of *Acacia nilotica* nodal explants *in vitro*

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Abstract
Major problems encountered in establishing axenic cultures are contamination and browning of explants and media. Contamination is initiated in explants excised from soil-borne tissues or adult trees. Several sterilizing agents (Tween 20, chlorine water, silver nitrate, mercuric chloride, etc.) or a wash in antibiotic solution are recommended to reduce this. . Establishing *in vitro* cultures of *Acacia nilotica* old tree nodal explants was a serious problem. The present study was undertaken to create a suitable protocol for *in vitro* micropropagation of nodal explants of *Acacia nilotica* subsp. *indica*. Contamination was controlled by sterilizing the explants by washing in polysan (5%, v/v along with 500mg/l PVP), thorough washing under tap water, 70% alcohol treatment and 0.1% $HgCl_2$ treatment. Plant tissues release phenolic substances through their cut ends, which turn the media dark brown and toxic. Addition of various antioxidants or/and transfer of explants to fresh medium twice or thrice, at a few days' intervals may overcome the problem. Collection of explants in antioxidant solution and a wash in antioxidant solution prior to inoculation was helpful in reducing the phenolic exudation in the present investigations. The addition of antioxidant (citric acid) to the medium also checked browning to some extent. The rate of infection and browning of explants varied in different seasons, the maximum being during winters and the minimum during summers of 2019. This was inversely related to the morphogenic response of explants i.e. maximum caulogenesis occurred *in vitro* during July (6.3±1.4 shoots per explant). The *in vitro* raised shoots showed 100% rooting on 2mg/l IBA augmented Nitsch's (N) medium.

Keywords: Boric acid (H_3BO_3), Mercuric chloride ($HgCl_2$), Polyvinylpyruvic acid (PVP), Seasonal variation

INTRODUCTION

The success of *in vitro* clonal propagation of trees has been restricted mainly due to their recalcitrant nature and other associated problems, such as heavy inborne as well as surface-bound infection, leading to less survival frequency and oxidation of phenolic substances leached out from the cut ends of explants. Seasonal variations in temperature, light intensity, humidity, and pathogen pressure can significantly influence these parameters, affecting the overall outcomes of tissue culture experiments (Mohammadi *et al*., 2011; Martini *et al*., 2013; Devi and Yadav, 2022). Elevated temperatures and increased humidity during certain seasons create favourable conditions for the proliferation of microbial contaminants such as bacteria, fungi, and yeasts (Alagarsamy *et al*., 2018; Huang *et al*., 2019). Contamination can lead to a loss of valuable germplasm, reduced growth rates, and poor morphogenic responses of explants during tissue culture (Permadi *et al*., 2023).

Additionally, the spread of pathogens can negatively impact the health and vigour of tissue-cultured plantlets. Contamination may occur initially at the culture's establishment stage or during later subcultures (Omamor *et al*., 2007; Silva *et al*., 2020). These contaminants may vary from bacteria, fungi, microorganisms, mites, and thrips (TheodosyMsgoya *et al*., 2012). Of these, the most serious is bacterial infection, which can be systemic and difficult to detect (Singh *et al*., 2011; Gioushy *et al*., 2020; Bhat *et al*., 2022). They may be present within the pith, sclerenchyma, xylem lumen or parenchyma tissues, causing plant culture losses and affecting the micropropagation efficiency.

Many bacterial genera act as contaminants, such as *Pseudomonas, Agrobacterium, Aerococcus*, *Xanthomonads*, *Acinetobacter, etc*. (Varghese and Joy, 2015). Contamination may be introduced due to insufficient heating or mishandling of instruments during inoculation (Yahia *et al*., 2019). In some cases, the bacteria become resistant to standard glassware sterilization techniques, but upon reaching the culture medium, these grow faster than the cultured tissue (Varghese and Joy,2015; Bhat *et al*., 2022).

Apart from contamination, another major problem encountered in culturing plant tissues *in vitro* is the browning of excised plant tissues and nutrient media. The severity of browning varies in different plants, species, tissues or organs, their developmental phase, age of tissue/organ, nutrient medium and a number of other variables (Huang *et al*., 2002 ;Tao *et al*., 2007;Gioushy *et al*., 2020; Bhat *et al*., 2022). Browning and necrosis are common problems in cultures *in vitro*. This results in the explant death. Depending on whether enzymes are involved or not in the browning processes, tissue browning can be characterized as enzymatic or non-enzymatic. The principal cause of explant browning in plant tissue culture is mostly enzymatic. Browning is primarily attributed to the oxidation of phenolic compounds catalyzed by enzymes like polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and peroxidase (POD) (Selvarajan *et al.,* 2008; Dobránszki and Teixeira da Silva, 2010). Phenols are released when the explants are cut (Beckman, 2000; Jones and Saxena, 2013). These are then oxidized to quinones, which form cross-links with proteins or result in tissue polymerization thereby forming dark melanin compounds that ultimately result in tissue disruption (Krishna *et al*., 2018). Thus, the browning inhibits growth of the explants and sometimes death. Environmental factors, such as light intensity and temperature, can also influence the activity of these enzymes and contribute to seasonal variations in browning. The present study was undertaken to create a suitable protocol for *in vitro* micropropagation of nodal explants of *Acacia nilotica* subsp. *indica*.

MATERIALS AND METHODS

Source of plant material

The plant material of *A. nilotica* subsp. *indica* was collected from 30 to 40-year-old trees growing in the fields or from 8 to 9-year-old plantations growing as avenue trees in Delhi, from January to December 2019. Young sprouts growing orthotropically from the tree trunk or shoots arising after coppicing were used as a source of plant material.

Culture media

Stock solutions were prepared by using analytical grade salts (E. Merck India Ltd., Bombay). Twenty to hundred times concentrated solutions were stored in a refrigerator (Dewan *et al*., 1992, Narang, 2022). The basal media were either used alone or supplemented with growth regulators such as (i) cytokinins as BA (N 6 -benzyladenine), Kn (Kinetin), 2iP[6 (Y,Y,dimethylallylamino)purine] and zeatin and/or (ii) auxins as IAA (indole-3- acetic acid), IBA (indole-3-butyric acid), NAA (a-naphthalene acetic acid), NOA (3-naphtyloxy acetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid). For rooting of *in vitro* raised shoots, the shoots were cultured on basal medium containing various auxins (IAA, IBA, NAA, NOA, 2,4 -D) at different concentrations (Shukla *et al*., 2023). The plantlets developed *in vitro* were transferred to a mixture of soil and sand (1:1) in small plastic pots. For their better climatization to soil, specific microbial strains (SR 353A, SR 165 and SR 166) were used.

The medium was occasionally adjuvanted with AC (activated charcoal), ascorbic acid or citric acid. The pH of the media was adjusted to 5.8 using 1Normal NaOH or 1Normal HCl, before autoclaving. The media were gelled with 0.8% agar (Qualigens, Bombay, India). As a source of carbon, 3% sucrose (Daurala, Delhi, India) was used for explant cultures.

Incubation of culture

In vitro cultures of *A. nilotica* old tree nodal explants were maintained in the culture room at a temperature of 25±2 °C, with 55±5% relative humidity under white fluorescent 450-640W cm 2 light emitted by 40W Crompton tubes creating 16 hour photoperiod.

Subculture

The nodal explants of *A. nilotica* were regularly subcultured at an interval of 25-30 days. Quick subculturing (at an interval of 15 days) was also carried out in a few experiments. In some experiments, the media were changed as soon as they turned brown (i.e., within 1-2 hours of inoculation) to check the browning of explants.

Recording of data

Observations were recorded at ten to twelve days intervals. The morphogenic response of explants was recorded in terms of (a) percentage of explants producing shoots/and roots (b) number of shoots/roots per explant and (c) shoot/root growth i.e., average length of shoots/ roots. The final data were recorded after sixty or ninety days of growth in culture; after that, the in vitro raised shoots were excised and subcultured for root induction.

Statistical analysis

As reported earlier for *Acacia holosericea* seedling explants (Narang, 2022; Shukla *et al*., 2023) and *A. nilotica* seedling explants (Dewan *et al*., 1992), in the present experiments also, the average number of shoots

per responding explant and the average length of shoots were represented as mean values along with standard error (Mean±S.E.). The percentage of responding explants was tested using the Chi-square method, considering variance at a 5 % level (Shukla *et al*., 2023).

RESULTS AND DISCUSSION

In vitro control of contamination

The use of several sterilizing agents such as tween 20, polysan, Bavistin, calcium hypochlorite, sodium hypochlorite, hydrogen peroxide, bromine water, chlorine water, silver nitrate, mercuric chloride, hydrogen peroxide, combination of antibiotics, timentin, streptomycin sulfate, and gentamycin etc. have been recommended by various workers (Varghese and Joy, 2015; Sarkar *et al*., 2019; Benisheikh, 2015; Kuppusamy *et al*., 2019; Sivanesan *et al*., 2021). A wash in antibiotic solution or culturing the explants on an antibiotic-containing medium has also been tried for severe infection (Gioushy *et al*., 2020; Bhat *et al*., 2022).

In the present experiments, the mature nodal segments were sterilized in two different ways, i.e., (a) Normally, the material (young offshoots growing orthotropically on tree trunk or coppiced shoots) was brought to the laboratory in an aqueous solution containing ascorbic and citric acids (50 mg/l each) plus polyvinylpyrrolidone (PVP, 500 mg/l). They were washed thoroughly under running tap water and then treated with 5% polysan solution (v/v, Polypharm Pvt. Ltd., Bombay) containing 500 mg/l of PVP for 20-25 minutes and again washed under running tap water. The material was dipped in 70% ethanol for 10 minutes and subsequently with 0.1 % mercuric chloride (w/v) for 10 minutes. A rinse followed this in autoclaved distilled water containing 50 mg/l each of ascorbic and citric acids plus 500 mg/l of PVP. Finally, the material was thoroughly washed with sterilized distilled water. During the period of maximum infection (December to March) the material was collected in 2% (w/v) picric acid solution. Thereafter, these were washed under running tap water and dipped in 5% polysan (v/v) containing 2-3 teaspoons full of Bavistin for 20-25 minutes and again washed under running tap water. These were surface sterilized with 70% ethanol for 10 min. and a 1-2 min. dip in 95% ethanol. Subsequently, the material was washed with sterilized distilled water containing 50 mg/l each of ascorbic acid, citric acid and 500 mg/l of PVP and then washed thoroughly with sterilized distilled water once again. The thus sterilized explants were inoculated under aseptic conditions in a laminar flow cabinet.

Control of browning

Since browning of explants was inhibitory for *in vitro* morphogenesis, a number of experiments were designed to prevent it. Several attempts have been made in different plant species to eliminate the problem. Experiments have been carried out by incorporating of oxidants into medium (Ndakidemi *et al.*, 2014; Shimelis *et al*., 2015; Cai *et al*., 2020), pre-soaking of explants in antioxidants (Singh *et al*., 2011), incubation of cultures in the dark (Dobranszki and Teixsiera, 2010), coating the lower ends of explants in molten paraffin wax, and frequent sub culturing of explants (Titov *et al*., 2006; Ahmad *et al*., 2016; Singh and Patel, 2016). However, the effectiveness of different methods varies with different plant species and their physiological conditions.

In the present investigation, explant browning was found to be related to seasonal variations (Fig. 1); the minimum occurred during summers (June to August) when the best response was elicited (Fig. 2). To minimize browning, the explants were collected in an aqueous solution containing 25 mg/l each of ascorbic and citric acids and 500 mg/l of PVP. After surface sterilization, they were washed with distilled water containing 50 mg/l each of citric and ascorbic acids plus 500 mg/l PVP. Soaking explants in antioxidant solution prior to sterilization or treating the explants with antioxidant solution has been reported to be helpful in reducing their browning (Chai *et al*., 2018; Irshad *et al*., 2018; Amente and Chimdessa, 2021). This explant collection and sterilization method checked the browning to some extent (Babaei *et al*., 2013).

Morphogenic response

Maximum morphogenic response of *A. nilotica* nodal explants was obtained in July, with 24% contamination and minimum explant browning. Similar reports by Sultana *et al*., 2020; Zafar *et al*., 2021. Healthy multiple shoots differentiated at all levels of Kn used. A maximum of 76% of explants developed an average of 4.1±0.9 shoots per explant at 0.5 mg/l Kn medium (Figs

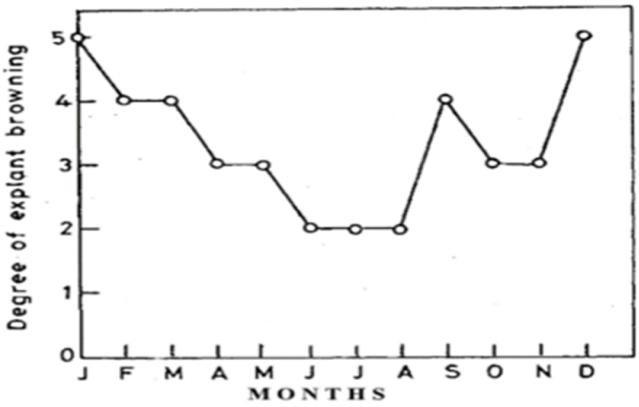

Fig. 1. *Seasonal variation in extent of browning of Acacia nilotica nodal explants; 1= little browning at cut ends, 2= browning of excised wounded tissue and partial browning of outer tissue of explant, 3= partial browning of outer tissue of explant and medium, 4= browning of the entire explant and medium, 5= complete browning and necrosis*

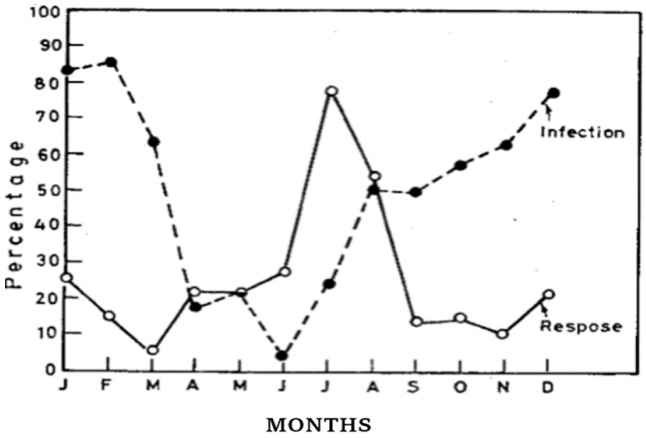

Fig 2. *Seasonal effect on morphogenesis of Acacia nilotica explants*

3A; Table 1). Shoots attained a length of 1.5±1.1 cm. Although the percentage of explants forming shoots was more (85%) at the lower level of Kn (0.1 mg/l), the average number of shoots per explant was higher at 0.5 mg/l. Basal medium alone showed 3.1±1.0 shoots per explant. Friable to compact yellowish-white calluses were induced on the surface of explants. Healthy multiple shoots differentiated at all levels of Kn used. However, browning of shoot tips was observed quite frequently.

Boron is known to prevent the defoliation of leaves by inhibiting shoot-tip browning (Wang *et al*., 2015). It is generally added to the nutrient medium as boric acid. Experiments were, therefore, carried out by varying the borate contents in N medium to optimize the exact levels required for multiple shoot induction and to prevent the browning of shoot tips. Explants were reared on shoot regeneration medium (N + 0.5 mg/l Kn + 100 mg/l c.a.) supplemented with 100-500 mg/l boric acid. Within 20- 25 days of inoculation, multiple shoot buds differentiated on nodes in almost all the media used. Among the varying concentrations of H_3B0_3 tried, a maximum average of 6.3±1.4 shoots per explant was recorded in 60% explants on shoot regeneration medium containing 100 mg/l H_3B0_3 (half of the original concentration used in N medium, i.e. 200 mg/l). The percentage of explants differentiating shoots was much higher (75) at 200 mg/l H_3B0_3 level, but reducing the H_3B0_3 concentration to 100 mg/l promoted caulogenesis, as it supported a higher number of shoots per explant as well as long healthy shoots with well-expanded leaves (Fig. 3B; Table 2). Further, it prevented the shoot tip from browning. Higher levels of boric acid (> 200 mg/l) showed a reduction in the frequency of explants developing shoots but increased explants showing calluses on their surface and at the cut ends.

Explant source, size and orientation

The morphogenic response of *A. nilotica* nodal explants was related to both the orientation and size of the explants, as also reported earlier in bamboo (Basto *et al*., 2012; Mudoi *et al*., 2014) . It was observed that only the explants inoculated vertically on semisolid medium showed bud-break, while others placed flat on the surface of the medium failed to show any response (Fig. 4A,B). Similarly, the size of the explant also played an important role in bud-break. Explants with diameters less than 0.3 cm did not develop any shoot buds (Fig.Aa;Bb), while thicker explants (0.3-0.5 cm diameter) easily differentiated multiple shoot buds (Fig. 4Ab;Bb). However, neither the length of the explant nor the number of nodes (serially from the tip) was significant in inducing morphogenesis. Best response was obtained in explants 1-2 cm long and 0.3-0.5 cm in diameter. The source from which the explants were excised was another important factor in their response *in vitro*. It was noted that only fresh shoots growing orthotropically on the tree trunk or shoots from coppiced trees responded under *in vitro* conditions, while the others did not.

Rooting of excised shoots and transfer of plantlets to soil

Axenically grown shoots (1-5 cm long) excised from nodal explants were cultured on Nitsch's (N) media supplemented with 1 -3 mg/l of different auxins like IA A, IB A, NAA and NOA. Roots were induced at the

Table 1. *In vitro* morphogenic response of *Acacia nilotica* nodal explants on 0.1-3 mg/1 Kn supplemented N medium. Explants were collected in the month of July

Kn (mg/l)	Number of explants	Explants forming shoots (%)	Average number of shoots per explant	Average shoot length (cm)
0	24	59[a*]	3.1±1.0	0.8±0.9
0.1	24	85[a,b]	2.7±1.2	0.8±0.9
0.5	24	76[b]	4.1±0.9	1.5±1.1
1.0	24	67[b]	2.7±1.4	0.7±0.6
2.0	24	42	2.6±1.4	0.7±0.9
3.0	24	25	2.7±1.1	0.3±0.1

[*]Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level.

Fig 3. ***(A)*** *Multiple buds developing on Nitsch's medium + 0.5mg/l Kn* ***(B)*** *Healthy shoots on N medium + 0.5mg/l Kn + 100mg/l Boric acid* ***(C)*** *Shoots developing roots directly from their base on N medium + 2mg/l IBA*

Table 2. Effect of boric acid supplemented to shoot regeneration medium (N + 0.5 mg/1 Kn +100 mg/1 c.a.) *in vitro* on morphogenesis of *Acacia nilotica* nodal explants collected in July

H_3BO_3 (mg/l)	Number of explants	Explants forming shoots (%)	Average number of shoots per explant	Average shoot length (cm)
N + 200	24	40	1.2±0.3	0.3±0.1
Kn + c.a. + 200	24	75	2.4±0.4	1.3±0.8
N + 0	24	20	1.7±0.8	1.0±1.1
Kn + c.a. + 0	24	18	1.5±0.8	0.8±0.1
N+ 100	24	25	2.4±0.8	0.8±0.2
Kn + c.a. + 100	24	60	6.3±1.4	1.4±1.3
N +250	24	30	1.8±0.6	0.2±0.1
Kn + c.a. + 250	24	23	1. 4±0.4	1.0±0.4
N+ 300	24	35	1.8±0.8	0.6±0.4
Kn + c.a. + 300	24	23	2.2±0.9	0.3±0.2
N + 350	24	25	2.2±0.7	1.2±0.9
Kn + c.a. + 350	24	9	1.0±0.0	0.2±0.0
N + 400	24	25	1.6±0.8	0.5±0.3
Kn + c.a. + 400	24	40	1.8±0.5	1.0±0.9
N+ 500	24	25	1.6±0.8	0.5±0.3
Kn + c.a. + 500	24	36	1.3±0.4	0.7±0.2

base of shoots in all the auxin-containing media, within 15-20 days of inoculation. However, medium supplemented with IBA at 2 mg/l concentration showed the best response. Sixty-one per cent of explants differentiated direct roots (without any intervening callus) in this medium (Fig. 3C). The roots varied from 0.2-3 cm in length.

The *in vitro* raised plantlets (1- 5 cm long), after 40-50 days of incubation on rooting medium, were transferred to small pots containing either (i) garden soil: sand (1:1), (ii) garden soil:sand: vermiculite (1:1:1), (iii) garden soil:sand: sawdust (1:1:1) or (iv) sand: sawdust (1:1).These were maintained under high humidity in the culture room by covering them with plastic bags and spraying them with distilled water 6-7 times in a day. However, when shifted from the rooting medium to the pots, the plantlets failed to thrive for more than 3-4 weeks due to severe defoliation within 20-25 days. Similar reports of defoliation were reported in *A. holosericea* (Shukla *et al*., 2023). In the present work, specific rhizobial strains (SR 353A, SR 165 and SR 166) helped the plants acclimate to the soil successfully.

Conclusion

Multiple shoots (4.1±0.9) from nodal explants of *A.nilotica* subsp. *indica* were induced in 76% of cultures on N medium supplemented with 0.5 mg/l Kn *in vitro*. However, in some cases, such shoots turned brown at their tips and showed poor growth with unopened

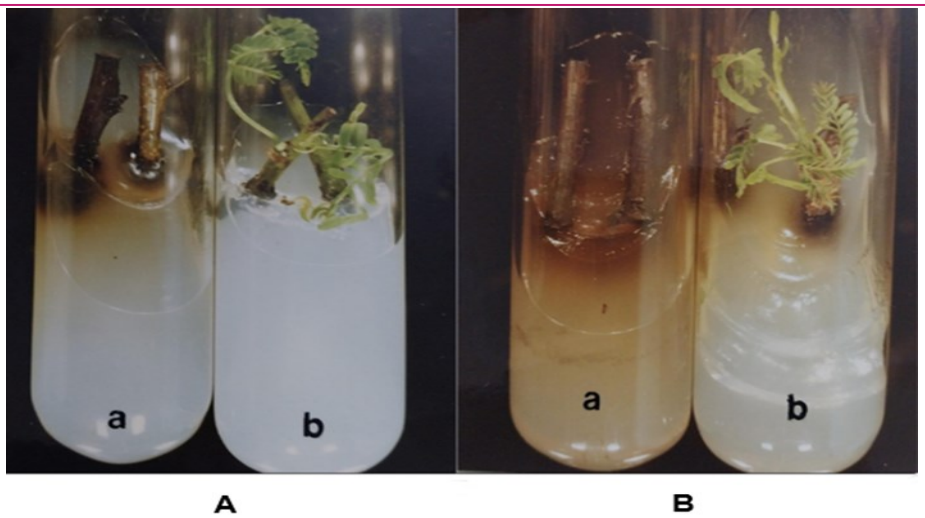

Fig 4 ***(A)*** *Explants placed vertically on the medium showing leaching: a-no bud break in explants <0.3cm, b-bud break in explants 0.3-0.5cm thick* ***(B)*** *Same, when placed horizontally showing excessive leaching and no morphogenic response: a-no bud break in explants <0.3cm, b-bud break in explants 0.3-0.5cm thick*

leaves. Lowering the concentration of boric acid from 200 to 100 mg/l in the Nitsch's medium helped to prevent the shoot tip browning, resulting in healthy development. Hence, N medium (containing 100 mg/l H_3BO_3) supplemented with 100 mg/l citric acid and 0.5 mg/l Kn was the optimum combination for the best caulogenic response in *A. nilotica* nodal explants. The plant material showed considerable seasonal variation in caulogenesis as recorded during different months of the year, best being in July, which coincided with minimum browning and infection. It was observed that only the explants inoculated vertically on a semisolid medium showed bud-break. In contrast, others placed flat on the surface of the medium failed to show any response. The present experiment demonstrates the micropropagation of *A. nilotica in vitro* through a highly efficient protocol. Since the tree has multipurpose uses and shows poor germination *in vivo*, the present investigation will be very useful for enhancing the growth of this species.

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Conflict of interest

The authors declare that they have no conflict of interests.

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