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In vitro Shoot Regeneration and Antioxidant Enzymes Activities of *Thevetianeriifolia* Juss

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Abstract: ***Thevetianeriifoliais one of the important plants because it's a good source of natural compounds that have therapeutically effects. The main aim of this study is to investigate the effect of plant growth regulators on in vitro callus induction and regeneration of shoots from it, as well as the estimation of antioxidant enzymes activities during these processes. Callus was induced from internodal explants by culturing them on MS medium contained BA(0.0, 1.0 or 2.0 mg/L) and IBA(0.0, 1.0 or 2.0 mg/L) , then shoot regeneration from callus was occurred. Results showed that the combinations of 1.0 mg/L BA+ 1.0 mg/L IBA, 1.0 mg/L BA+ 2.0 mg/L IBA, 2.0 mg/L BA+ 1.0 mg/L IBA and 2.0 mg/L BA+ 2.0 mg/L IBA were the best for callus induction (100%). The highest regeneration frequency (100%) and large mean of shoots regeneration (7.29) were achieved in MS medium supplemented with 1.0 mg/L BA + 0.0 mg/L IBA. A positive relationship was observed between regeneration capacity and antioxidant enzymes activities in which the highest levels of CAT and SOD activities were reached during the stages of shoots regeneration from callus.***

Key words: Antioxidant enzymes, callus, *Thevetianeriifolia*, *in vitro*, shoot regeneration.

Abbreviations: ANOVA: analysis of variance; BA: N-6-benzyl adenine; CAT: catalase; IBA: indole-3-acetic acid; LSD: least significant difference; MS: Murashige and Skoog medium; ROS: reaction oxygen species.

1. Introduction

*Thevetianeriifolia*Juss.(yellow oleander) is a member of the family Apocynaceae, which is an important ornamental and medicinal plantthat distributed worldwide in tropical and subtropical regions (Joshi, 2000). The other synonyms of this plant is *Thevetiaperuviana*Pers.K.Schum (Van Beek*et al.*, 1984). *T. neriifolia*is a good source of natural compounds that are used as pharmaceuticals such asthevetin, cardiac glycosides, kaempferol, quercetin, neriifolinand other flavonoids that are known to be strong antioxidants and showing anti-inflammatory activity, anti-microbial activity, anti-cancer and cardio-tonic activity (Zavaleta, 2012; Save *et al.*, 2015).

The conventional method for propagation of *Thevetia*species is through seeds, which is time consuming due to poor germination. Thus, using of plant tissue culture technique to propagate this plant can be applied to help in rapid propagation, germplasm conservation and production of secondary metabolites (Zibbu and Batra, 2010). *In vitro* shoot regeneration of *T. neriifolia* has been established from an organogenesis pathway via callus formation from leaf, roots, immature seeds and stem segments (Taha*et al.*, 2010; Nesy*et al.*, 2015). The successful of *in vitro* plant morphogenesis associated with the high efficiency of metabolism which resulting in the production and accumulation of reactive oxygen species (ROS) that causing oxidative stress for plant cells and tissues (Benson, 2000; Mittler*et al.*, 2004; Libik-Konieczny*et al.*, 2012 ; Zur*et al.*, 2014). These ROS are produced as byproducts of normal metabolic processes occurring in chloroplasts, peroxisomes, mitochondria and cytoplasm (Mittler, 2002; Apel and Hirt, 2004; Konieczny*et al.*, 2014). Therefore, plants produce well developed antioxidative systems to protect plant cells and tissues against the oxidative stress caused by the toxic levels of ROS. The antioxidative systems consist of several enzymes like Superoxide dismutase (SOD), Catalases (CAT) and Peroxidases (POX) (Mittler, 2002; Batkova*et al.*, 2008; Gantait*et al.*, 2011).

During the recent years, various reports were achieved in different plants to study the relationship between *in vitro* organogenesis and the changes of antioxidant enzymes activities (Libik*et al.*, 2005; Meratan*et al.*, 2009; Rout and Sahoo, 2013; Konieczny*et al.*, 2014 ; Tubic*et al.*, 2016). The present study was intended to determine the callus induction and plant regeneration capacity of *Thevetianeriifolia*Juss. and to survey the changes of antioxidant enzymes activities during these processes.

2. Materials and Methods

Preparation of Plant Material

Internodes of *Thevetianeriifolia*Juss. were used as explants for callus induction and regeneration. Young branches were taken from five years old trees, and after removing of leaves, they were cut into pieces (4 cm approximately) and washed with liquid soap under running tap water and they were used as a source of explants. In the laminar air flow cabinet, they were surface-sterilized in 70% (v/v) ethanol for 1 min, then transferred into 3% (v/v) of sodium hypochlorite solution for 15 minutes, and finally, they were rinsed three times with sterile distilled water.

Culture medium and incubation conditions

For callus induction, internodal explants were cut into pieces (about 1.5 cm in length) and cultured horizontally on MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with benzyl adenine (BA) at concentrations (0.0, 1.0 or 2.0 mg/L) in combination with indole-3-butyric acid (IBA) at concentrations (0.0, 1.0

or 2.0 mg/L). The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving. The molten medium was distributed to suitable glass tubes and about 15 ml for each tube. Then, the poured medium was autoclaved at 121 ºC and 1.04 kg/cm² pressure for 20 minutes. All cultures were incubated under 16 hrs. photoperiod (1000 lux provided by cool-white and day light fluorescent lamps) in growth room at 25 ± 2 ºC. The observations of callus induction were taken daily and recorded weekly . The frequency of callus induction on explants was calculated (number of explants induced callus/ total number of explants * 100).

Regeneration conditions

When callus induction was occurred on explants, six-weeks old callus was sub-cultured on to a fresh MS medium supplemented with the same combinations of BA and IBA that mentioned above for testing callus shoot regeneration potential. Percentage of shoot regeneration (number of cultured induced shoot/ total number of explants incubated * 100) and mean number of shoots were scored after five weeks of incubation. Each experiment was repeated two times and all experiments were carried out of seven replicates.

Antioxidant enzyme activities

1g of fresh callus was homogenized using a mortar and pestle with 10 ml of phosphate buffer (50 mM, pH= 7), 50 mM ethylene-diamine-tetra acetic acid (EDTA) and 10% polyvinyl-polypyrrolidone (PVP). The whole extraction procedure was carried out on ice. The homogenates were centrifuged at 10000 rpm for 10 min. at 4 ºC, and the obtained extract was used for estimation of enzymes activities.

Catalase (CAT) activity was estimated according to Aebi (1984). The reaction buffer solution consisted of 2ml of phosphate buffer (50 mM, pH= 7), 2ml of H_2O_2 solution (30 mM) and 40 µl of enzymatic extract. The volume of interaction should be 4.04 ml incubated at 25 ºC for 1 min. then absorbance was measured at 240 nm. The activity of CAT was calculated by following equation:

Catalase activity(unit) = (Δbs/min * reaction volume)/ 0.01

Where: Δbs: absorbance difference within a minute; 0.01: constant; min: reaction time; 4.04ml: reaction volume.

Superoxide dismutase (SOD) activity was estimated according to Marklund and Marklund method (1974). The reaction buffer solution composed of 2ml of Tris buffer solution (pH=8.2), 0.5 ml of pyrogallol (0.2 mM) and 50 µl of enzymatic extract. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of pyrogallol oxidation per minute.

The activity of SOD was calculated by the following equation:

SOD activity (unit) = { (%inhibition of pyrogallol reduction/50%)*reaction volume} / Total test period.

Statistical Analysis

A completely randomized design was used. The experiments were repeated two times and the data for each parameter were subjected to analysis of variance (ANOVA). Significant differences were assessed using least significant difference (LSD) test at P≤0.05 (GenStat, 2012).

3. Results and Discussion

Callus Induction:

The internodal explants began to develop callus after ten days of cultivation. Among the tested combinations of BA and IBA in MS medium for callus induction , results in Table-1 showed that 1.0 and 2.0 mg/L of BA were the best for callus induction (95.2 and 90.5 % respectively), whereas the highest percentage of callus induction was observed in 1.0 and 2.0 mg/L of IBA (81.0 and 90.5 respectively) as compared with control (52.4%). Results also showed that the maximum callusing rate (100.0%) was found in the combinations: 1.0mg/L BA+1.0 mg/L IBA, 1.0 mg/L BA + 2.0 mg/L IBA, 2.0 mg/L BA + 1.0 mg/L IBA and 2.0mg/L BA + 2.0 mg/L IBA as compared with other combinations, while the control (MS medium without any addition of BA and IBA) did not respond to induce callus. In tissue culture techniques, the formation of callus may be important for indirect organogenesis or for production of secondary metabolites, so that the type and concentration of cytokinins and auxins in the culture medium are the most important factors to stimulate callus formation in different plants (George *et al.*, 2008; Lubaina and Murugan, 2012; Dalila*et al.*, 2013; Rout and Sahoo, 2013; Nesy*et al.*, 2015).

Table 1: Effect of different concentrations of BA and IBA on callus induction percentage(%) from *Thevetianeriifolia*internodal explants

BA(mg/L)	IBA(mg/L)			Mean of BA
	0.0	1.0	2.0	
0.0	0.0	42.9	71.4	38.1
1.0	85.7	100.0	100.0	95.2
2.0	71.4	100.0	100.0	90.5
Mean of IBA	52.4	81.0	90.5	
LSD(0.05)	BA= 19.62		IBA= 19.62	
	BA * IBA= 33.98			

4. Shoots Regeneration

The frequency of indirect shoots regeneration was depending on the type and concentration of growth regulators added to the regeneration medium (Table-2-). For shoot regeneration, the embryogeniccalli which were greenish in color were separated from the explants remains and transferred into new fresh MS medium containing the same combinations of BA and IBA that mentioned previously (Figure-1). The highest frequency of shoot regeneration (81.0 %) was observed in MS medium containing 1.0 mg/L BA, whereas the medium that free from IBA gave 61.9 % as compared with other concentrations of the auxin. Callus gave the maximum shoot formation (100.0 %) in the medium containing the combinations of : 1.0 mg/L BA + 0.0 mg/L IBA and 1.0 mg/L BA + 1.0 mg/L IBA. Generally , the frequency of shoot regeneration may require cytokinins only or in some cases in combinations with auxins (Radhika*et al.*, 2006; Meratan*et al.*, 2009; Martins *et al.*, 2015).

Table 2: Effect of different concentrations of BA and IBA on shoot regeneration percentage(%) from callus of *Thevetianeriifolia* .

BA(mg/L)	IBA(mg/L)			Mean of BA
	0.0	1.0	2.0	
0.0	0.0	0.0	0.0	0.0
1.0	100.0	100.0	42.9	81.0
2.0	85.7	42.9	28.6	52.4
Mean of IBA	61.9	47.6	23.8	
LSD(0.05)	BA= 20.13 IBA= 20.13 BA * IBA= 34.86			

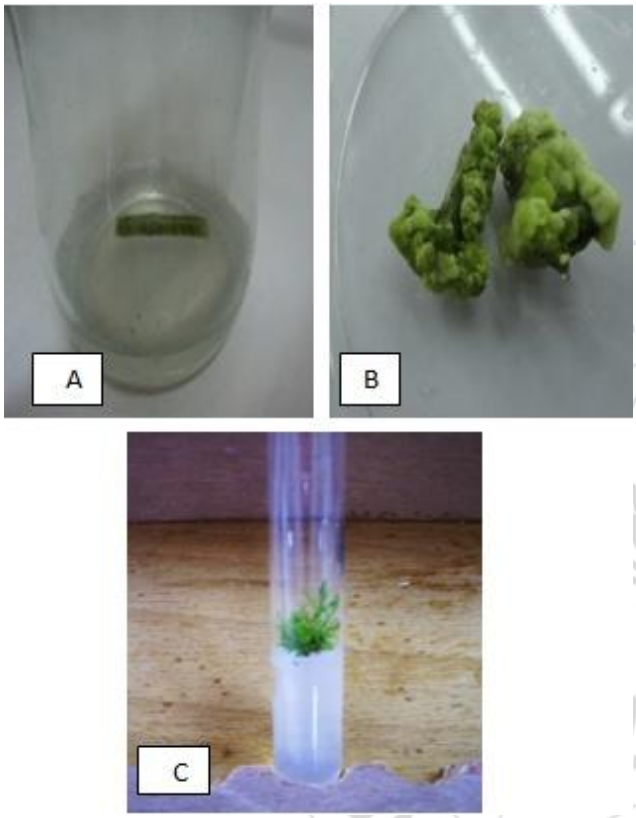

Figure 1: A) *Thevetianeriifolia*internodal explant cultured on MS medium. B) Callus covered the explants. C)Regeneration of shoots from callus on MS medium +1.0 mg/L BA.

The number of shoots proliferated per callus varied with the combinations of plant growth regulators used in the medium (Table- 3). The callus cultured on MS medium without any growth regulators did not give any shoot proliferation. It was observed that medium containing 1.0 mg/L BA gave the maximum shoot number (3.24), whereas it was lowest at other cytokinin concentrations. Similar findings were obtained by Kavani*et al.* (2011) in *Matthiolaincana*, Haq*et al.* (2013) in *Vincarosea* and Rout and Sahoo (2013) in *Elephantopusscaber* who found that the addition of cytokinins at suitable concentrations could be stimulate shoot formation from callus. On the other hand, medium without any addition of the auxin IBA gave the highest shoot number (3.81) as compared with other IBA concentrations. In case of interaction between BA and IBA, the maximum number of shoots (7.29) was obtained when callus was cultured in MS medium supplemented with 1.0 mg/L BA only, but the inclusion of IBA in the medium reduced significantly the shoots number. This revealed the superiority of cytokinins over auxins in shoot formation, while auxins stimulate callus induction, somatic embryogenesis and root formation (Khanam*et al.*, 2000). Researchers reported that inclusion of auxins in low concentrations with cytokinins could increase the shoots proliferation (Jain and Ochatt, 2010; Nesy*et al.*, 2015 ; Tubic*et al.*, 2016).

Table 3: Effect of different concentrations of BA and IBA on shoot number per callus derived from internodal explants of *Thevetianeriifolia* .

BA(mg/L)	IBA(mg/L)			Mean of BA
	0.0	1.0	2.0	
0.0	0.0	0.0	0.0	0.0
1.0	7.29	1.71	0.71	3.24
2.0	4.14	2.14	0.29	2.19
Mean of IBA	3.81	1.29	0.33	
LSD(0.05)	BA= 0.905 IBA= 0.905 BA * IBA= 1.567			

5. Antioxidant Enzymes Activity

As shown in Table- 4 , the enzymes activities were low in callus and increased in shoot regeneration phase. Catalase (CAT) activity was 26.72 units/g tissue during the first 10 days of cultivation and proliferation of callus, followed by a slight decline, but not significantly after 20 days of culture to reach 24.14 units. Significantly increasing was occurred in CAT activity with the proceeding of organogenesis and shoot formation to reach the highest levels at 60 and 70 days of culture (31.40 and 33.73 units respectively). The changes in superoxide dismutase (SOD) activity were shown also in Table- 4. After 10 days of culture, the SOD activity was 4.43 units, which then decreased significantly to reach 2.07 units after 20 days of culture. After that , SOD activity tended to increase significantly with the developmental stages of callus and shoot regeneration and remained in high level (4.71 units) at 70 days of culture. Similar results were observed in *Aloe barbadensis*(Hu *et al.*, 2003), *Acanthophyllumsordidum*(Merata*et al.*, 2009) and *Gardenia jasminoides*(Sayd*et al.*, 2010).

This study is the first of antioxidant enzymes activities in *Thevetianeriifolia*Juss., most reports demonstrated that callus secreted antioxidant enzymes such as CAT and SOD during the stages of callus differentiation and suggested that these enzymes were involved in the processes of shoot regeneration where the metabolism pathways were active and resulting in large amounts of oxidative radicals (Tian*et al.*, 2003; Gantait*et al.*, 2011; Filipovic*et al.*, 2015). CAT is an important antioxidant enzyme known to play a role in decomposition of H_2O_2 and helped to protect tissues from highly reactive hydroxyl radicals (Krishnaraju*et al.*, 2009). Therefore, significant increasing in CAT activity during the shoot regeneration than in callus stage may suggest the efficient scavenging mechanism of this enzyme to eliminate H_2O_2 that produced in regenerated shoots (Merata*et al.*, 2009 ; Xu*et al.*, 2013). The increasing in SOD activity could be associated to the processes of callus proliferation and shoot regeneration which revealed the capacity of the enzyme to protect the tissues from damages of oxidative stress caused by superoxide radicals (Tian*et al.*, 2003; Libik*et al.*, 2005; Rout and Sahoo, 2013 ; Tubic *et al.*, 2016).

Table 4: Effect of *Thevetianeriifolia*culture age and morphogenesis on antioxidant enzymes (CAT and SOD) activities *in vitro*.

Culture age (days)	CAT (Units)	SOD (Units)
10 *	26.72	4.43
20 *	24.14	2.07
30 *	27.66	4.12
40 **	27.75	3.72
50 **	29.21	3.36
60 ***	31.40	4.55
70 ***	33.73	4.71
L.S.D.(0.05)	**4.902**	**1.551**

* Callus only (friable white), ** Organogenesis callus (granular greenish) and *** Less callus with shoots.

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