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THIDIAZURON INDUCES *IN VITRO* BUD BREAK AND SHOOT DEVELOPMENT FROM NODAL EXPLANTS OF ORTHOTROPIC SHOOTS OF MAIDENHAIR TREE (*GINKGO BILOBA* L.)

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ABSTRACT

In the present study, axillary shoot bud induction, the growth of shoot and callus formation was observed. Shoots were cut to prepare nodal explants (5 - 10 mm long), surface sterilized and inoculated on MS (Murashig and Skoog, 1962) medium supplemented with different concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4 or 5 µM) of TDZ for 24 days. Highest bud break (100%) was obtained at 0.005 µM TDZ after 14 days of initial culture. The same cultures were further maintained and subsequently obtained with 20.6 mm shoot length and 7.2 average number of leaves for another 10 days. Similarly, all other TDZ's concentrations below 1 µM were also effective in bud breaking and development of shoots with green and vigorous growth. The frequency of bud break was significantly reduced with the increasing concentrations of TDZ. There were 2 phases of callus formation on the explant, i.e., before bud break and after the break. The frequency of callus induction was highest (100%) at 5µM TDZ before bud break along the nodal region. The rate of callus formation was significantly reduced once bud began to break down and initiation of green axillary shoots. The shoots were further developed, rooted and acclimatized to glasshouse conditions. We demonstrated that axillary buds of *G. biloba* could be initiated and proliferated with TDZ under *in vitro* conditions.

Keywords: Bud break; Callus; Ginkgo; Nodal explant; Thidiazuron

1. INTRODUCTION

Propagation thirst of medicinal and aromatic plants is being increased in industry, academia and health sciences of the world (Camper *et al*., 1997). The commercial preparation of medicines involves diverse processes comprise various side effects on human and animal health. Therefore, public is reverting towards conventional medication and likely becoming interested in the propagation of medicinal plants for personal as well as for sale to stallholders. This necessitates rapid multiplication of such plants by using some reliable approaches.

Ginkgo biloba is a gymnosperm belongs to the family Ginkgoaceae. It is a medicinal as well as ornamental plant reported as a living fossil dating back to 270 million years used in traditional medicines (Choudhury and Das, 2014). *G. biloba* is native to China and the only living representative of the late Mesozoic period. Many alkaloids including ginkgolides in the root and in leaves are found in *G. biloba* (Hobbs, 1998). This is a slow growing and recalcitrant tree needs up to 20 years to reach maturity (Hobbs, 1998). Rapid propagation methods thus need to establish to gain its commercial production. *In vitro* propagation of *Ginkgo* has been reported from cultures of embryo, shoot tip and callus (Bekhit *et al*., 2008).

Adenine based cytokinins are important stimulators for *in vitro* shoot growth through axillary bud break of

different tree species (Bowen-O'Connor *et al*., 2007; Llorente and Apostolo, 2012; Li *et al*., 2015). Similarly, 6-Benzylaminopurine (BAP) and Kinetin have been reported for micropropagation and multiplication from diverse explants of *Ginkgo* (Mantovani *et al*., 2013). Phenyl urea type cytokinins such as thidiazuron (TDZ) has been reported a potent growth regulator for tree micropropagation (Huetteman and Preece, 1993) as well as *in vitro* axillary shoot proliferation and regeneration of various other plants and tree species (Akram and Aftab, 2008, 2016; Aftab, 2012; Kher *et al*., 2016). However, there is a lack of information in the contemporary literature about how to use TDZ for *in vitro* bud break of *Ginkgo*. Thus, aim of the present study was to determine the effect of various TDZ concentrations on bud break for axillary shoot proliferation from mature nodal explants of *G. biloba*.

2. MATERIALS AND METHODS

Vertically growing green shoots (orthotropic shoots) of the current season were selected from a tree growing in PU Botanical Garden, University of the Punjab, Lahore.

Tissue Culture Medium was prepared after adding the essential nutrients of MS (Murashige and Skoog 1962) salts and vitamins, 3% sucrose and required amount of plant growth regulator into the measuring flask. After this, pH of the medium was adjusted 5.80, solidified with 0.8% agar and poured 10 ml in each culture tube. The tubes were capped with a piece of polyethene sheet and tied with rubber bands, labeled and autoclaved at 114 kPa and 121 °C for 15 min. The medium was taken out from the autoclave and stored at culture room conditions of 16 h light (35µm $m^{-1}s^{-1}$) and 8 h dark period at 25 ± 2 °C until use.

Shoots from mature tree were cut with a razor and brought to the lab for the preparation of 1-2 cm long nodal explants of *Ginkgo*. The explants were disinfected with v/v 15% commercial bleach (Robin, Procter & Gamble, Pakistan) for 10 min followed by w/v mercuric chloride (0.1% $HgCl_2$) for another 10 min. After this procedure explants were then thoroughly washed with autoclaved distilled water thrice.

The dead tissues on both explant edges were removed and cultured on MS medium supplemented with 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4 or 5 µM TDZ for bud break and axillary shoot initiation. The data for percent bud break were recorded after 14 days, whereas the data for shoot length and number of leaves was observed after 24 days from the initial culture of bud break. Morphological characteristics of the developing shoots were also recorded.

For root induction, half MS medium fortified with 5, 10 or 15 µM each of indole-3-butyric acid (IBA) + α-naphthalene acetic acid (NAA) was used added with activated charcoal (0.1%). The data for root induction was collected after 35 days of initial culture. The rooted shoots were then taken out from the culture tubes washed under running tape water and treated in a 0.1% solution of Bavistin fungicide. The plantlets were shifted in plastic pots (10 cm × 15 cm) containing sterilized peat moss and placed in the growth room conditions for 45 days. Then moderately acclimatized plantlets were then shifted in the large (8 × 10 inch) earthen pots in the glasshouse for further acclimatization.

Statistical analyses were performed using ANOVA then Duncan's multiple range test (DMRT) was done at $p<0.05$. R^2 was used to check the correlation between treatments and undesirable callus formation as well as between auxin and root induction. All tests

were performed by using SPSS 16.0.

3. RESULTS AND DISCUSSION

3.1 Shoot growth and development

Nodal explants from current year sprouts of mature tree showed good results in terms of little contamination as well as high rate of *in vitro* culture establishment. Bud break started after 5 days of explant culture at 0.001 µM TDZ (Table 1, Fig.1A,B) or 0.005 µM TDZ after 10 days (Figure 1C). Highest (100%) bud break was obtained at 0.005 µM TDZ with 20.60 mm shoot length and 7.22 mean number of leaves after 24 days of initial culture (Figure 1 D, E). Vigorous, broad leaves, little swelling of explant's base was observed on the same medium. However, explants on other media got necrotic due to leaching and explant's browning.

Our results demonstrated that very little amount (0.001 µM) of TDZ was highly effective for bud breaking of *Ginkgo* nodal explants. The effect of TDZ has been reviewed in many woody plants for *in vitro* axillary and latent bud induction dating back of 24 years (Huetteman and Preece, 1993). Since the discovery of TDZ replaced other cytokinins because of its effectiveness in micropropagation of woody plants (Ahmad and Faisal, 2018). With the increasing dose of TDZ in the medium, bud break, as well as other shoot parameters, significantly reduced in the present study. Moreover, high amount of TDZ intend to swell the explants and no bud break or browning of explants occurred at its higher concentrations (Table 1). This may be due to its high cytokinin activity in *in vitro* cultures (Huetteman and Preece, 1993). Moreover, TDZ produces callus at the base of *in vitro* growing shoots above its optimum level. Such phenomenon was commonly observed in our previous work of teak (Akram and Aftab, 2008) as well as in other plants (Bhusarea *et al*., 2018).

There were two phases of undesirable callus formation, before and after bud breaking of explant. The proportion of both calluses was significantly increased with the increasing amount of TDZ (Figure 2). The callus formed before bud break was detrimental in terms of delaying axillary shoot growth. This is a negative phenomenon observed during the present investigation that may be due to the need for subsequent shoot growth. However, once axillary bud initiated, callus seemed beneficial that made it possible for the availability of essential nutrient's for subsequent shoot growth. Callus may also be the source of various totipotent cell initials for multiple shoot formation. Our results are consistent with our previous study as the little callus formation appeared essential for multiple shoot formation in teak (Akram and Aftab, 2008).

3.2 Rooting of shoots

Shoots were rooted on different concentrations of IBA + NAA (Figure 3 A, B). The rate of rooting was highest (38.5%) with 1.8 mean numbers of roots and 15 mm root length was obtained at 15 µM of both the combination of IBA + NAA after 35 days of initial culture (Figure 3 C, D, E). The rooting parameters were highly correlated ($R^2 = 0.9288$) with the rooting hormones. Rooted shoots were acclimatized in the glasshouse with fare rate (50%) of survival. Generally, both are the strong auxins induced rooting in apple and various other plant species (De Klerk *et al*., 1999).

The present study demonstrated the possibility of micropropagation from nodal explants of orthotropic green shoots of *G. biloba* using TDZ as a strong plant growth regulator.

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Table 1: Effect of TDZ on *in vitro* shoot induction from nodal explants of *G. biloba*

TDZ (µM)	Bud Break % After 14 days	Shoot length (mm) after 24 days	Number of leaves after 24 days	Culture's morphology
0.001	60.55 ± 5.55^{cd}	18.22 ± 6.33^{b}	3.22 ± 1.23^{bc}	Callus formed beneath the emerging shoot
0.005	100^{a}	20.60 ± 4.21^{a}	7.22 ± 2.22^{a}	Vigorous, broad leaves, little swelling of explants at the base
0.01	50.12 ± 4.21^{cde}	17.22 ± 5.32^{b}	1.05 ± 0.45^{cd}	Swelling at the shoot base
0.05	60.45 ± 5.33^{cd}	15.32 ± 4.25^{bc}	2.32 ± 1.22^{bcd}	Medium browning
0.1	80.55 ± 4.45^{b}	13.45 ± 3.12^{bcd}	2.45 ± 0.81^{bcd}	Medium browning
0.5	33.33 ± 4.25^{def}	12.36 ± 4.21^{cd}	1.12 ± 0.33^{cd}	Medium browning
1	31.25 ± 3.33^{def}	11.42 ± 3.33^{cde}	0^{e}	Leaching exudates
2	25.45 ± 2.41^{ef}	11.08 ± 3.02^{cde}	1.02 ± 0.04^{cd}	Leaching exudates
3	21.22 ± 2.10^{ef}	10.36 ± 2.36^{cde}	1.42 ± 1.04^{cd}	Shoot tip necrosis
4	0^{f}	0^{e}	0^{e}	Explant browning
5	0^{f}	0^{e}	0^{e}	Explant browning

30 explants were used and the experiment was repeated thrice. Different letters over means indicate significantly different results by Duncan's test at $p\leq0.05$.

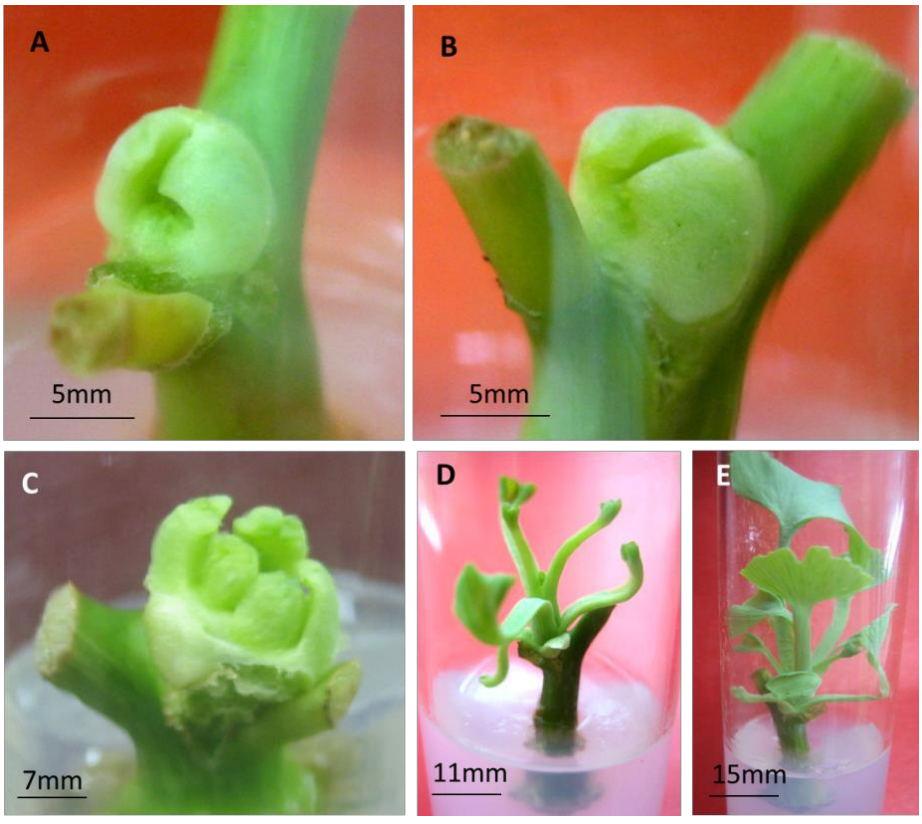

Figure 1 A-E: Effect of various levels of TDZ on bud breaking and shoot development of *G. biloba*.

A, B. Bud break and formation of leaf buds on MS + 0.001 µM TDZ after 5 days of initial culture. **C.** Further development of the same cultures at 0.005 µM TDZ after 10 days. **D, E.** Elongation and formation of leaves of an axillary shoots after 15 and 24 days old cultures, respectively at 0.005 µM TDZ.

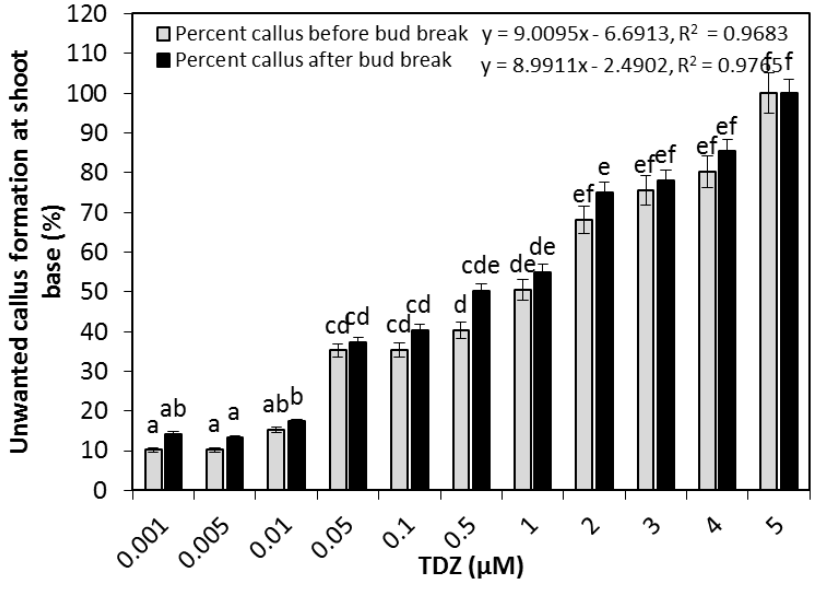

Figure 2: Undesirable callus formation during *in vitro* bud breaks of *G. biloba* affected by various levels of TDZ. Outside bars indicate ±SE. Each value represented by small letters is the significant results obtained by Duncan's multiple range test $p \leq 0.05$.

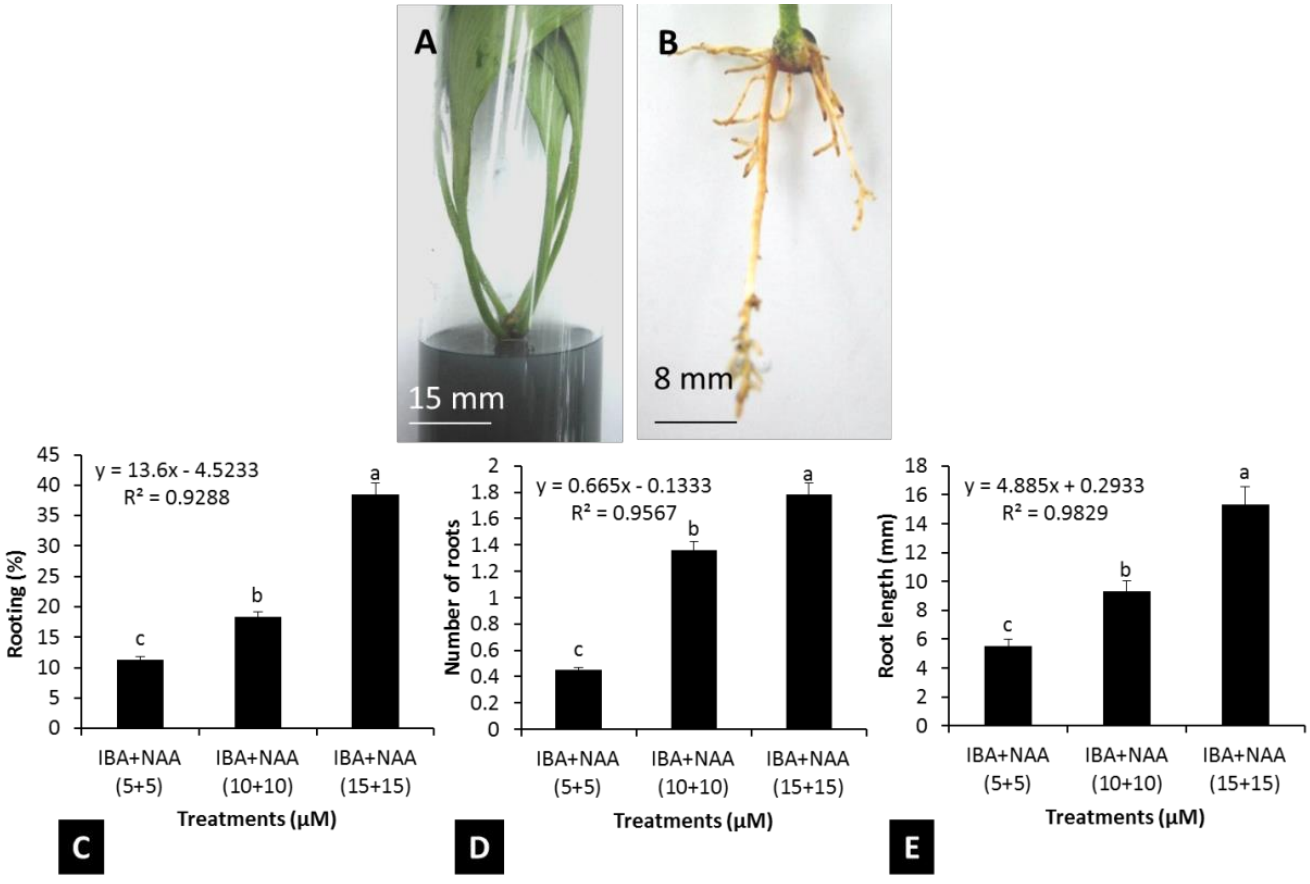

Figure 3 A-E: *In vitro* rooting of shoots of *G. biloba* after 35 days of initial culture. Bars over columns indicate ±SE. Each value represented by the same letters is not significantly different by Duncan's multiple range test $p \leq 0.05$. **A.** Rooting of shoot in ½ MS + 15 µM IBA + 15 µM NAA + 0.1% charcoal after 35 days. **B.** Larger view of rooting zone. **C**. Graphs showing rooting %, **D**. Number of roots and **E**. Root length.