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Prevention of shoot tip necrosis, hyperhydricity and callus production associated with in vitro shoot culture of *Ulmus glabra*

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ABSTRACT: *Ulmus glabra* is economically important forest species, greatly valued for its timber quality and its landscape. Unfortunately it is in danger of extinction due to Dutch Elm Disease. Therefore, an efficient micropropagation method is of considerable importance for breeding and genetic improvement of elm. Explants of two mature donor trees of *Ulmus glabra* investigated for the influence of medium, composition media and growth regulators on proliferation in terms of physiological disorders include apical necrosis, callus production and hyperhydricity which may cause trouble with in vitro shoot proliferation. Low level of Topolin reduced significantly these abnormities; however BAP especially in combination with TDZ worsened them. Compared to MS medium, best results were obtained on the modified ME medium (supplemented with 0.2 mg l^{-1} Topolin, 10 mg l^{-1} glutamine, 100 mg l^{-1} sequestrene and double concentration of $MgSO_4$ and KH_2PO_4). Modified ME significantly increased elongation and improved vigor of in vitro shoots.

***Keywords*:** micropropagation; Topolin; medium; apical necrosis; Wych elm.

INTRODUCTION

Wych elm (*Ulmus glabra*) is a hardwood elm tree, highly valued for its interesting quality of timber as well as its cold and pollution tolerance (Conde et al 2008). Unfortunately, susceptibility of elm trees to Dutch Elm Disease (DED) has been caused a severe decline in their populations in recent decades (Stipes 2000). DED is provoked by a vascular wilt fungus *Ophiostoma novo-ulmi* (Et-Touil et al. 1999). Consequently, such devastation in population size has been decreased genetic diversity followed by increasing the genetic vulnerability of the trees. Therefore research studies concerning in situ or ex situ conversation of elm germplasms and the modern breeding programs have been conducted (Gartland et al. 2001; Fenning et al. 1996). However, this method can be only feasible after the optimization of micropropagation of elm species (Chalupa 1994; Gartland et al. 2000; Mala 2000; Biroscikova et al. 2004).

Various micropropagation methods have been reported for elm species such as adventitious shoot formation from nodal segments in *U. minor* (Conde et al. 2008), *U. glabra* (Biroscı´kova´ et al. 2004), *U. pumila* (Dorion et al. 1987).

Recently micropropagation of *Ulmus glabra* was carried out by Biroscikova et. al (2004). They mentioned that Thidiazuron (TDZ) in combination with 6-benzylaminopurin (BAP) gave the higher rate of shoot multiplication than the most optimal BAP treatment. In *Ulmus minor*, Conde et al.(2004) achieved the best proliferation rate with DKW (Driver and Kuniyuki 1984) basal medium supplemented with 4.4 µM BAP and 0.05 µM Indole-3-Butyric Acid (IBA). Despite many protocols on elm micropropagation, proliferation efficacy is strongly influenced by the genotype and physiological age of donor tree (Rodríguez et al. 2004). In the other hand, some undesired physiological disorders like vitrification, shoot tip necrosis or basal callus production, have been reported during the in vitro proliferation of wych elm (Biroscicova et al. 2004). Vitrified tissues are characterized by translucent and glassy appearance. This

problem has been attributed to the a number of factors associated with culture medium including the high NH4 levels, high cytokinin concentrations and low K levels (singha, 1990).

Shoot tip necrosis (STN) is another physiological problem occurred in in vitro cultured plants. As a result of STN, apical shoot becomes brown and dies. It has been reported that STN can be affected by the type and concentration of cytokinin, calcium and boron levels (Bairu and Jain, 2009b). Reduction of endogenous cytokinins in the apical shoots contributes to STN (Kataeva et al. 1991). Application of aromatic cytokenin, meta-topolin, riboside, significantly reduced STN in *Harpagophytum procumbens* cultures (Bairu et al., 2009c). Topolins, especially meta-topolin have been used for cultutre initiation, optimization of tissue culture protocols at various stages including multiplication, rooting, and acclimatization as well as increasing the quality of in vitro plants, hyperhidricity alleviation and reduction of STN (Adeyemi et al., 2012).

In this research, two different young genotypes of wych elm were selected for micropropagation. The effects of different cytokinins and their concentrations, various culture media and compounds on proliferation in order to reduce vitrification, chlorosis, STN and callus production rates were investigated.

MATERIALS AND METHODS

Plant materials and culture initiation

Two young donor trees of *Ulmus glabra* (G1 and G2) have been selected for sampling. Stem cuttings with axillary buds were collected during growth season. Before establishment, stems were surface sterilized according to Biroscikova et al (2004). They were first immersed in dishwashing liquid solution and rinsed under tap water for 30 min to remove surface dirt, then sterilized by agitation in 0.1% peracetic acid containing two drops of tween for 15 min. The buds were further rinsed four times with autoclaved water supplemented with 500 mg l^{-1} citric acid for 15 min. They were then cut into 2-3 cm segments containing two nodes. Finally scale leaves of buds were removed by means of sterilized pincers and scalpel. The excised segments were established in culture medium. Four weeks after establishment, in vitro elongated shoots were cut and used for proliferation experiments.

Culture media

Woody Plant Medium (WPM; Lloyd and McCown 1980) was used as culture medium in establishment stage. Young buds (which had no scale leaves) were established on free hormone WPM but decoated buds were cultured on WPM supplemented with 0.4 mg l^{-1} BAP.

For proliferation experiments, elongated shoots were transferred to different media treatments as following:

WPM medium supplemented with 0.4 mg l^{-1} BAP in combination with TDZ (0 and 0.05 mg l^{-1});

MS (Murashige and Skoog, 1962) supplemented with different levels of BAP (0 and 0.4 mg l^{-1});

MS supplemented with different levels of Topolin (0.1, 0.2, 0.3, 0.4 mg l^{-1}) in combination with TDZ (0 and 0.05 mg l^{-1});

Modified ME (Cos and Frutos 2000) (with doubled concentrations of $MgSO_4$ و KH_2PO_4) supplemented with 0.2 mg l^{-1} Topolin, 10 mg l^{-1}glutamine and 100 mg l^{-1} Sequestrene (FeEDDTA).

MS medium containing two different chelating agents; FeNaEDTA (37 mg l^{-1}) and 100 mg l^{-1} Sequestrene.

Treatments were supplemented with 37 mg l^{-1} NaFeEDTA chelating agent but in some mentioned treatments, sequestrene was used. Media were solidified with 5.7 g l^{-1} agar (Duchefa). The pH of media was adjusted to 5.7 with 1 M NaOH. In all of the experiments, the general conditions of the growth chamber were 24±2°C and a 16/8-h (day/night) photoperiod with light provided by fluorescent lamps at a photon flux intensity of 2000 lux.

Data recording and statistical analysis

All treatments consisted of four replications. In vitro proliferation rate, shoot length, node number and rooting percentage were recorded four weeks after culture for each treatment. Additionally the rates of STN, vitrification, chlorosis and callus production were determined and recorded. Data were subjected to analysis of variance using a completely random design. The means of treatments were compared with Duncan's multiple range test (DMRT) to

distinguish differences between treatment means at the probability level of 0.05 using SPSS for windows, version 16.

RESULTS AND DISCUSSION

Result:

Sterilization with 0.1 mg l^{-1} peracetic acid reduced contamination rate to 9%. However, sterilization with Sodium hypochlorite (NaOCl) (2%) resulted in a contamination rate of 80-100% (data not shown).

Furthermore, establishment of young buds (having no scale leaves) on WPM containing 0.4 mgl^{-1} BAP caused callus production and release of phenolic compound, leading to death of buds. So young buds established in WPM free hormone. Decoated buds however, only sprouted on WPM containing 0.4 mgl^{-1} BAP.

In vitro elongated shoots (achieved from decoated buds or young buds) were subcultured on various treatments and showed different responses to the culture conditions.

Subcultured explants of both genotypes on WPM media (supplemented with 0.4 mg l^{-1} BA in combination with 0 and 0.05 mg l^{-1} TDZ) resulted in vitrified shoots and produced more calli as well as STN was happened and led to shoot death compared to the other treatments (Table 1). Moreover in presence of TDZ these three abnormities worsened and atypical adventitious shoot produced. Therefore WPM was not used for proliferation experiments anymore.

MS medium supplemented with 0.4 mg l^{-1} BAP decreased vitrification, STN and callus production compared to WPM supplemented with 0.4 mg l^{-1} BAP.

MS free hormone, MS free hormone supplemented with 100 mg l^{-1} sequestrene and modified ME supplemented with 0.2 Topolin, 10 glutamin and 100 sequestrene caused no vitrification, STN and callus.

According to Table 1, Topolin in low concentration in MS medium significantly decreased callus production and STN rates and vitrification were not happened. However, increase of its concentration raised those disorders. Addition of TDZ intensified vitrification, STN and callus production (Figure 1).

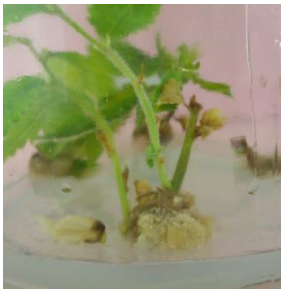

Figure 1. Callus production of G2 explants in presence of high level of Topolin (0.3 mgl^{-1})

In an attempt to find out the auxin-like activity of chelating agants, two different Fe sources were used in MS free hormone, 37 mg l^{-1} NaFeEDTA and 100 mg l^{-1} Sequestrene. Results showed that 87.5% of G1 explants rooted in treatment of Sequestrene but unfortunately G1 explants were omitted because of internal contamination. 50% of G2 explants and 21% of G1 explants produced roots in presence of NaFeEDTA (Table 1). Application of sequestrene significantly improved leaf chlorosis.

Table 2 strongly suggests that the genotype and Topolin concentration have interacting effects. Average shoot length and average node number were also affected by genotype as G2 had longer shoots and more nodes in MS free hormone medium. Increasing Topolin in medium caused longer shoots and more nodes in G1.

Culturing G2 explants on modified ME supplemented with 0.2 mg l^{-1} Topolin and 100 mg l^{-1} Sequestrene resulted in average shoot length of 3.59 cm and average node number of 4.06 which were significant compared to all other treatments in G2 (Table 2). Moreover, there was no sign of apical necrosis or hyperhydricity, only a negligible percentage of callus production (<10%) (Table 1). It should be noted that, G1 shoots were omitted in some treatment, due to internal contamination.

Table 1. The effects of different cytokinins or medias on proliferation of Ulmus glabra. Callus production, apical necrosis, hyperhydricity, chlorosis and root production rates were compared. Callus production, apical necrosis, hyperhydricity and chlorosis rates were similar for both genotypes. G1 explants which were omitted, showed by "O"

medium	Composition media Or Plant Growth Regulators	Cal. Pr.	Api. Nec.	Hyp. Hyd.	Chl.	Root Production(%) G1	G2
WPM	0.4 mg l^{-1} BAP	++	++	+++	++	–	–
WPM	0.4 mg l^{-1} BAP + 0.05 mg l^{-1} TDZ	+++	+++	+++	++	–	–
MS	0.4 mg l^{-1} BAP	+	++	±	+	–	–
MS 0	–	–	–	–	+	25%	50%
MS 0	(100 mg l^{-1} Sequestrene 300 Fe)	–	–	–	–	O	87.5%
MS	0.1 mg l^{-1} Topolin	+	±	–	+	–	–
MS	0.2 mg l^{-1} Topolin	+	±	–	+	–	–
MS	0.3 mg l^{-1} Topolin	++	+	–	+	–	–
MS	0.4 mg l^{-1} Topolin	++	+	±	+	–	–
MS	0.1 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	++	+	±	+	–	–
MS	0.2 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	++	+	±	+	–	–
MS	0.3 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	+++	++	+	+	–	–
MS	0.4 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	+++	++	+	+	–	–
Modified ME	0.2 mg l^{-1} Topolin + 100 mg l^{-1} Sequestrene 300 Fe + 10 mgl^{-1} glutamin	±	–	–	–	O	–

Table 2. Effects of different media, plant growth regulators and composition media on axillary shoot proliferation, adventitious shoot proliferation, average shoot length and average node number. Statistical data compared in four parts. Each part separated by line . G1 explants which were omitted, showed by "O"

medium	Composition media	Average shoot length (cm) G1	G2	Average node number G1	G2
MS	0.4 mg l^{-1} BAP	2.62b	4.39ab	2.4b	4.39a
MS free	37 mg l^{-1} NaFeEDTA	2.59b	5.05a	3.30ab	4.85a
MS	0.1 mg l^{-1} Topolin	2.06abc	2.63abc	2.25ab	3.66ab
MS	0.2 mg l^{-1} Topolin	2.81abc	2.04abc	3.58ab	2.37ab
MS	0.3 mg l^{-1} Topolin	1.87abc	2.46abc	0.92b	3.04ab
MS	0.4 mg l^{-1} Topolin	3.70abc	2.83abc	4.78a	3.39ab
MS	0.1 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	1.55abc	0.93bc	2.35ab	1.68b
MS	0.2 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	4.87a	1.31abc	3.16ab	1.93ab
MS	0.3 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	4.50ab	1.64abc	4.70a	1.95ab
MS	0.4 mg l^{-1} Topolin + 0.05 mg l^{-1} TDZ	4.16ab	0.39c	4.62a	0.458b
Modified ME	0.2 mg l^{-1} Topolin + 100 mg l^{-1} Sequestrene + 10 mgl^{-1} glutamin	O	3.59a	O	4.06a
MS	0.2 mg l-1 Topolin	--	2.04b	--	2.37b

DISCUSSION

Several studies have revealed that plant growth regulator's pathways are connected with different complex networks include developmental and physiological processes (Bari and Jones, 2009; Aremu et al., 2012a). Surveys such as those conducted by Werner et al, (2001), Criado et al, (2007) and Aremu et al, (2012a) have shown the positive and negative roles of cytokenins on different processes related to growth factors.

Shoot tip necrosis is a common disorder and a leading cause of plant death characterized by browning of apical buds and leaves (Bairu et al. 2009c; Kataeva et al., 1991). The most common elements that are typically associated with STN are plant growth regulators especially commonly used cytokenins like BA and TDZ (Il'ina et al. 2006). Perhaps the most serious disadvantage of BA is that the formation of metabolite 6-benzylamino-9-b-D-glucopyranosylpurine is the contributing factor in some physiological abnormities and detrimental effects (Bairu et al. 2011b). Bairo et al, (2011b) and (2009b) observed that the rate of STN increased by higher concentrations of cytokinins.

In contrast to earlier survey conducted by Biroscikova et al. (2004) on proliferation of *Ulmus glabre*, however, in this study no evidence of healthy proliferation was detected by 0.4 mg BAP in combination with TDZ because of high rate of basal callus, atypical adventitious shoot, shoot tip necrosis and hyperhydricity.

Recent evidences suggest that Topolins can be a good replacement for commonly used cytokinins (BA or TDZ) in many plant tissue culture protocols. They can also improve some physiological disorders (Aremu et al., 2012a; Aremu et al., 2012b) and decrease the rate of STN (Amoo et al., 2011; Bairu et al., 2009c)

According to Table 1, BA and higher levels of Topolin; especially in combination with TDZ, caused high rate of STN but low rate of STN was observed in lower concentrations of Topolin. Interestingly, there was no STN symptoms in ME medium supplemented with 0.2 mgl $^{-1}$ Topolin. ME medium has higher concentration of calcium. Debergh (1988) reported that the analysis of necrotic apices showed deficiency of calcium.

A comparison of different cytokenins in this study revealed that there was a clear trend of decreasing in basal callus size at low levels of Topolin. Similarly Bairu et al, (2009b) observed less basal callus using of Topolin in micropropagation of *Harpagophytum procumbens*. Callus formation was not observed in explants cultured in MS free hormone; however rooting was induced. This is consistent with Thimann and Takahashi (1958) report which highlighted the role of chelating agents as auxin synergists. Basal callus formation might be as a result of auxin content at the end of explants which in presence of CKs can induce cell proliferation in the shape of callus (Marks and Simpson, 1994). Basal callus production in presence of cytokinins is more common in those species with strong apical dominance (preece et al. 1991).

Furthermore explants which were treated by Topolin produced more vigorous shoots than BAP treatments (Fig. 2). Aremu et al. (2012a) demonstrated that Topolin treated plantlets significantly were more vigorous than those treated with BAP.

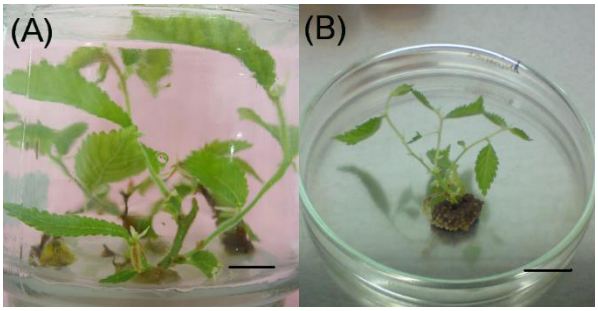

Figure 2. The effect of Topolin and BAP on explants' vigor. a explants treated with 0.2 mg l^{-1} Topolin after four weeks. b explants treated with 0.4 mg l^{-1} BAP after four weeks. Bar: 1 cm

By using 100 mg l^{-1} sequestrene (as iron source) in presence of low concentration of Topolin, the vigor of explants were increased. Moreover no sign of chlorosis was observed. Clapa et al, (2007) reported similar effect of sequestrene in tissue culture of blueberry.

Modified ME supplemented with 0.2 mg l^{-1} Topolin improved plant vigor and growth factors compared to other media (Table 1). ME medium has high ratio of NO_3 to NH_4 and high concentration of Ca^{++}. Chevre *et al,* (1983) state that chestnut shoot cultures grew and proliferated best in MS medium modified by doubling the usual levels of calcium and magnesium.

It was shown that low ratio of NO_3:NH_4 results in vitrification (Barker and Corey, 1987; Mott et al., 1985). The negative correlation between Ca ion concentration and shoot tip necrosis incidence has been found previously (Barghchi and Alderson, 1996; Debergh, 1988). However the average level of phosphate in ME medium was lower than MS. That's why double concentration of phosphate has been used. Considering that the level of phosphate which introduced in plant culture medium was up to 19.8 mM (Hall and klerk, 2008). However, MS medium contains 1.25 mM and many reports note that such level may be too low for plant culture (Hall and klerk, 2008).

Our results showed that shoots are sensitive to higher levels of cytokenins. Therefore, 10 mg l^{-1} glutamine was added for compensating lack of reduced nitrogen and reducing cytokenin requirement.

Multipication rate was highest in MS free medium following growth on MS supplemented with 0.4 mg l BAP (maximum 5 shoots per explants). However, Topolin did not increase multiplication rate. Mala et al (2013) reported increasing multiplication rate as twice in MS medium supplemented with Topolin following a subculture on MS supplemented with 0.5 BAP. They also recorded multiplication rates in treatments after 12 weeks. We suggest that the high multiplication rates in MS free hormone medium or in Topolin is related to BAP residues in explants from previous subcultures but not Topolin. By the same token in this survey, Aremu et al, (2012a) reported the inhibitory effect of high levels of Topolin on proliferation rate.

Additionally two genotypes had different response to treatments in view of node number and shoot length. G1 and G2 had more node number due to presence of TDZ and BAP respectively. Therefore node number and shoot length were genotype dependent.

Our results showed that in vitro shoots are sensitive to the level of cytokenins and the response is genotype dependent too. For future studies, the effect of modified ME supplemented with BAP needs to be investigated on micropropagation of Ulmus glabra.

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