

EFFECT OF CYTOKININ, SUCROSE AND NITROGEN SALTS CONCENTRATIONS ON THE GROWTH AND DEVELOPMENT AND PHENOLICS CONTENT IN *Magnolia* × *soulangiana* ‘Coates’ SHOOTS *in vitro*

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Abstract. Phenolics are believed to inhibit the shoot formation in magnolia *in vitro*. The aim of this study was to determine the influence of sucrose, nitrogen salts and cytokinin concentrations on the phenolics content in relation to shoot formation in *Magnolia* × *soulangiana* ‘Coates’ *in vitro*. The results showed that the concentration and ratios of benzylaminopurine (BAP), sucrose and nitrogen salts in the Murashige and Skoog (MS) medium had a significant effect on the leaf and axillary shoot formation as well as on the phenolics content. The highest multiplication rate (4.8 shoots/explant) and shoots of good quality were obtained on medium containing 0.2 mg·dm⁻³ BAP, 100% nitrogen salts in relation to the MS medium and 20 g·dm⁻³ sucrose. At this sucrose level, increasing BAP concentration from 0.2 to 1.0 mg·dm⁻³ resulted in the inhibition or slight stimulation of shoot formation depending on the nitrogen levels (100 and 75/50%, respectively). At low sucrose-to-nitrogen ratio in the medium, increased BAP levels induced the leaf browning. The highest inhibition of *M. × soulangiana* ‘Coates’ shoot formation has been observed on medium containing 30 g·dm⁻³ sucrose, reduced nitrogen salts levels and BAP at concentration 1.0 mg·dm⁻³. A medium with a high sucrose-to-nitrogen ratio stimulated also phenolics production in magnolia shoots. The addition of BAP lowered phenolics production compared with the control medium. At high sucrose-to-nitrogen ratio, increasing BAP levels significantly stimulated phenolics production. The results of the study showed that not in all the treatments did the enhanced phenolics levels in the shoots of *M. × soulangiana* ‘Coates’ coincide with decreased shoot formation.

Key words: axillary shoots, BAP, leaf browning, magnolia, sucrose-to-nitrogen ratio

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INTRODUCTION

The genus *Magnolia* L. (*Magnoliaceae*) consists of over 250 species and numerous hybrids and varieties that can be cultivated in temperate and tropical climates worldwide. As ornamental plants, they are grown because of their beautiful flowers of various colours, size, shape, fragrance and durability. They are recommended for planting in gardens and urban green areas. Less vigorously growing species and varieties can also be grown in containers for decorating department stores, theater foyers, conservatories, entrances to various buildings. New hybrid varieties are also notable for high resistance to frost. *Magnolia* × *soulangiana* Soul.-Bod. is a hybrid valued for its large flowers and early blossoms. Propagation of magnolia is difficult. It is usually carried out by specialized nurseries with many years' experience in propagation by layering and stem cuttings. Propagation of magnolia *in vitro* is an opportunity to increase the efficiency of the multiplication of these attractive plants. Currently, this method of propagating magnolia is being developed and improved [Czekalski 2007].

It is known that some species and cultivars of magnolia have different culture media requirement [Kamenická and Lanakova 2000]. The successful growth and development of axillary buds has been obtained on various basal salts media, including Standardi and Catalano [1985], Murashige and Skoog [1962], and Chée and Pool [1987] Vitis medium [Gabryszewska 1997, Kamenická and Lanakova 2000, Parris et al. 2012, Sokolov et al. 2014]. Among the growth regulators tested, BAP has been found to be more effective than *meta*-topolin, 2iP and TDZ for axillary multiplication in different *Magnolia* genotypes [Parris et al. 2012, Radomir 2012]. Although there are few reports on *M. × soulangiana* micropropagation [Gabryszewska 1997, Kamenická and Lanakova 2000, Podwyszyńska et al. 2000, Radomir 2012, Sokolov et al. 2014], many cultivars of this species have proven to be recalcitrant. The main problems in the propagation of magnolias *in vitro* are browning of explants, vitrification and low activity of axillary buds [Biedermann 1987, Kamenická et al. 1996, Mata-Rosas et al. 2006, Wojtania et al. 2012, Sokolov et al. 2014].

Axillary shoot multiplication of magnolias has been reported to be difficult because of the presence of phenolic substances [Biedermann 1987, Kamenická et al. 1996]. The study on *Magnolia* 'Ann' indicated, that media supplemented with phenolics binding agents (PVP and AC) produced greener leaves and increased in shoot length, but they reduced shoot multiplication and root initiation [Parris et al. 2012]. It has been found that phenolics are frequently affected by several internal and external factors, including light, the C/N ratio and growth regulators [Singh et al 1998, Giannakoula et al. 2012]. To our knowledge, there is no information on the amounts of phenolics in magnolia shoots depending on the growth conditions and the relationship between the phenolics level and shoot formation.

The aim of this study was to determine the influence of nitrogen, sucrose and cytokinin concentrations on the phenolics content in relation to shoot formation in *Magnolia* × *soulangiana* 'Coates' *in vitro*.

MATERIALS AND METHODS

Shoot cultures of *M. × soulangiana* 'Coates' were initiated from apical and axillary bud explants, collected from two-year-old field-grown plants. The initial cultures of explants and subsequent subcultures of shoots were performed on the basic Murashige and Skoog (1962) medium containing macro- and microelements, 100 mg·dm⁻³ myo-inositol, nicotinic acid, pyridoxine and thiamine (1.0 mg·dm⁻³ each), 1.0–1.5 mg·dm⁻³ benzylaminopurine (BAP), and solidified with 6.5 g·dm⁻³ commercial agar. The pH of the medium was adjusted to 5.6 before autoclaving. After the ninth subculture, the single shoots were separated and used in the experiment.

The influence of nitrogen salts (KNO₃, NH₄NO₃) present at different ratios in relation to the MS medium (100:100, 75:50), sucrose concentrations (20, 30 g·dm⁻³), and BAP concentrations (0.0, 0.2, 0.5, 1.0 mg·dm⁻³) on shoot formation and phenolics production by *M. × soulangiana* 'Coates' were studied.

The shoot cultures were kept at 23°C, under a 16/8 h day/night photoperiod provided by cool-white fluorescent lamps at 40 μmol m⁻²s⁻¹ (Philips TLD 36W/95). The single shoots were subcultured at 5 week intervals. The observations and measurements were recorded after 2 subcultures on the same medium. The number and length of the shoots, the number of leaves, including the number of necrotic leaves (%), as well as the amounts of phenolics in the shoots were determined. Each treatment consisted of 25 explants. The experiment was repeated twice (2 series).

To estimate the phenolics content, approx. 100 mg of plant tissue was homogenized in 1.5 ml of 80% ethanol and centrifuged at 2800 rpm for 20 min. The supernatant was mixed with 20% Na₂CO₃ and Folin-Ciocalteu reagent [Singleton and Rossi 1965]. The absorbance (λ = 760 nm) of samples was estimated spectrophotometrically on a microplate reader (Synergy 2, Bio-Tek, Winooski, VT, USA) according to Singleton and Rossi [1965] with modifications. The total phenolics content was calculated as milligrams of chlorogenic acid per 1 g of tissue fresh weight.

Data were subjected to analysis of variance and the means were compared by Duncan's test at the α = 0.05 significance level.

RESULTS

Shoot growth and development. As shown in Fig. 1, BAP significantly stimulated shoot formation in *M. × soulangiana* 'Coates' *in vitro*, but its effect depended on the nitrogen and sucrose levels. The shoots grown on cytokinin-free medium exhibited enhanced shoot length (fig. 1) and a low number of leaves (fig. 2) with large blades. On the MS medium containing 100% nitrogen salts, the most effective axillary multiplication (4.8 shoots/explant) was observed in the presence of BAP at the lowest concentration (0.2 mg·dm⁻³) and sucrose at 20 g·dm⁻³. At this sucrose level and nitrogen at 100% strength, increasing BAP concentration from 0.2 to 1.0 mg·dm⁻³ resulted in inhibition of shoot formation by 35%. In case of shoots growing in the presence of reduced level of nitrogen salts (75% strength of KNO₃ and 50% strength of NH₄NO₃ in relation to the MS) and sucrose at concentration 20 g·dm⁻³, axillary multiplication was the same effec-

tive at all BAP levels, reaching the value 4.5–4.7 shoots/explants. At both nitrogen levels, sucrose at 30 g·dm⁻³ significantly inhibited the shoot formation in *M. × soulangiana* ‘Coates’. On medium containing 30 g·dm⁻³ sucrose and reduced nitrogen salts, increased BAP concentration significantly decreased the axillary shoot formation (fig. 1). In the presence of high sucrose (30 g·dm⁻³) and nitrogen levels, the multiplication rate was similar at all BAP levels (3.6–3.4 shoots/explant). In those treatments, the longest shoots were also observed (fig. 1).

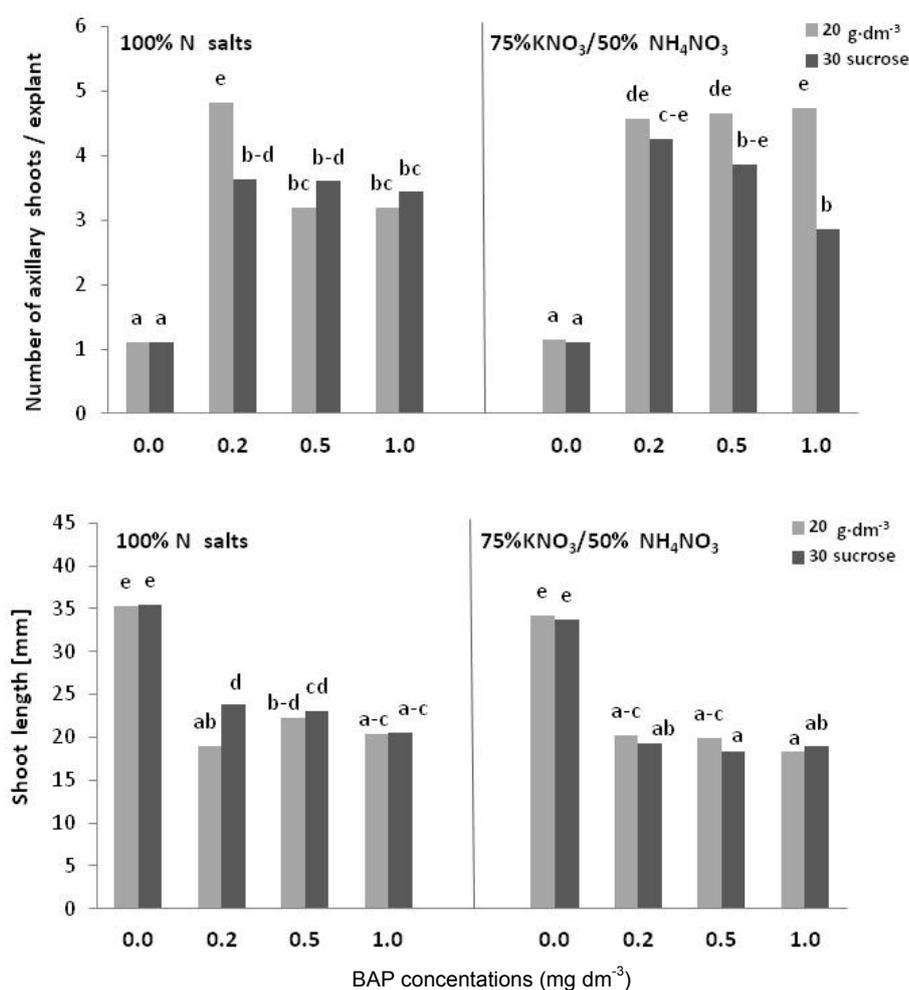


Fig. 1. The effect of different nitrogen levels (in relation to MS medium) and BAP and sucrose concentrations on shoot formation and shoot length in *M. × soulangiana* ‘Coates’ after a 5-week subculture period. Means of each growth parameter indicated with the same letter do not differ significantly ($\alpha = 0.05$) according to Duncan’s test

The results of our study showed that the interaction of cytokinin, nitrogen and sucrose had also a regulatory effect on the leaf formation and leaf browning in *M. × soulangiana* 'Coates' (fig. 2). In the presence of 100% nitrogen salts, the leaf formation varied according to BAP and sucrose levels. It was observed, that BAP supply significantly reduced or stimulated the leaf formation depending on the sucrose level (20 and 30 g·dm⁻³ respectively). On medium with reduced levels of nitrogen salts, sucrose at 30 g·dm⁻³ significantly inhibited the leaf formation independent of cytokinin levels.

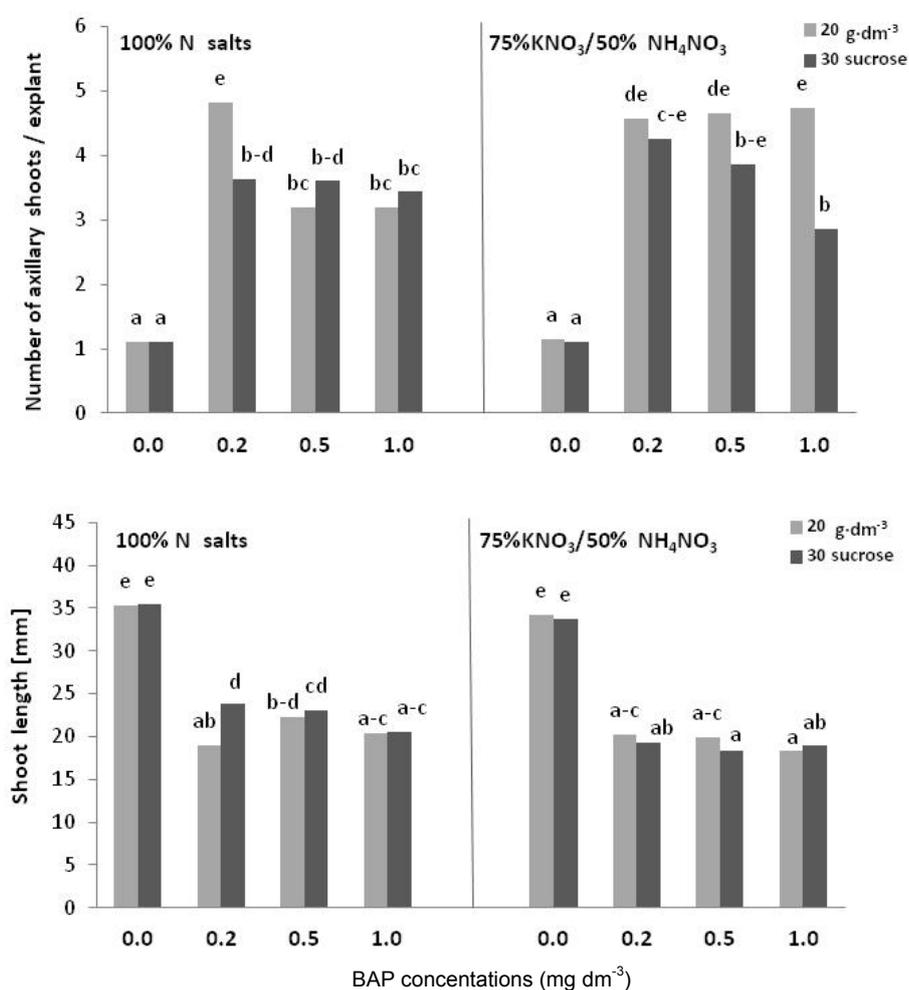


Fig. 2. The effect of different nitrogen levels (in relation to MS medium) and BAP and sucrose concentrations on leaf formation and the number of browning leaves in *M. × soulangiana* 'Coates' after a 5-week subculture period. Means of each growth parameter indicated with the same letter do not differ significantly ($\alpha = 0.05$) according to Duncan's test

It has been found that *M. × soulangiana* ‘Coates’ shoots cultured *in vitro* had tendency to leaf browning (fig. 2, 3). On cytokinin-free medium, the leaf browning was higher on medium containing 100% nitrogen salts. At this nitrogen level and $20 \text{ g} \cdot \text{dm}^{-3}$ sucrose, increased cytokinin concentration in the medium enhanced the leaf browning by 70% (fig. 2). However, cytokinin supply in the presence of high sucrose ($30 \text{ g} \cdot \text{dm}^{-3}$) inhibited the leaf browning (fig. 2). On medium with reduced nitrogen salts level, the leaf browning was proportional to the cytokinin and sucrose levels.

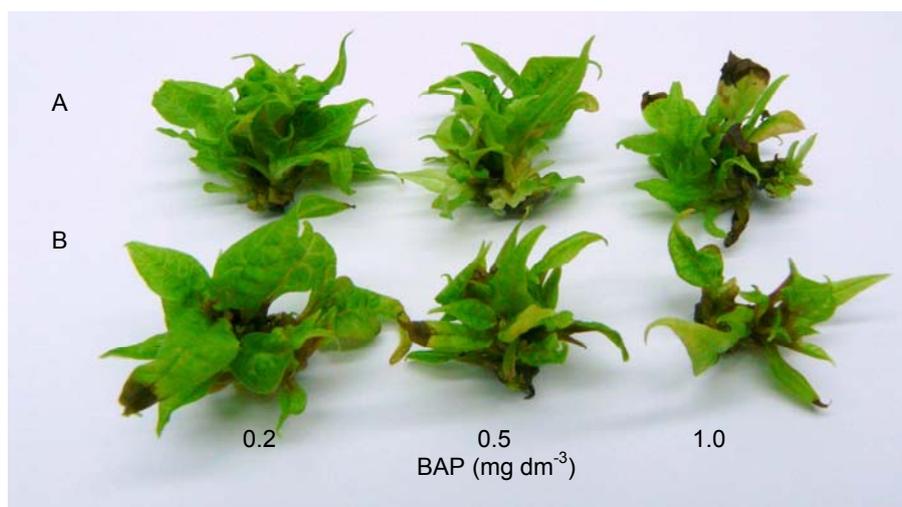


Fig. 3. Shoots of *Magnolia × soulangiana* ‘Coates’ after a 5-week subculture period on media supplemented with reduced amounts of nitrogen salts (KNO_3 at 75% and NH_4NO_3 at 50% strength of the MS medium), different BAP levels and sucrose at concentrations of $20 \text{ g} \cdot \text{dm}^{-3}$ (A) or $30 \text{ g} \cdot \text{dm}^{-3}$ (B)

Phenolics content. The shoots of *M. × soulangiana* ‘Coates’ growing under tissue culture conditions exuded to the medium a brown pigment on different growth media (data not shown). The measurements of the amounts of phenolics in magnolia shoots showed that their production was dependent on the level and ratio of cytokinin, nitrogen and sucrose in the medium (fig. 4). In general, increasing sucrose concentration from 20 to $30 \text{ g} \cdot \text{dm}^{-3}$ resulted in enhanced phenolics production. Moreover, higher phenolics content in magnolia shoots was observed at the reduced level of nitrogen salts than on the media containing nitrogen at 100% strength of the MS medium. At both nitrogen levels, cytokinin lowered phenolics production in magnolia shoots compared with the control medium. However, increasing BAP levels (0.2 – $1.0 \text{ g} \cdot \text{dm}^{-3}$) resulted in enhanced phenolics production. On medium containing the reduced levels of nitrogen salts and sucrose at concentration $30 \text{ g} \cdot \text{dm}^{-3}$, magnolia shoots produced the same amount of phenolics in the absence of cytokinin and in the presence of BAP at the highest concentra-

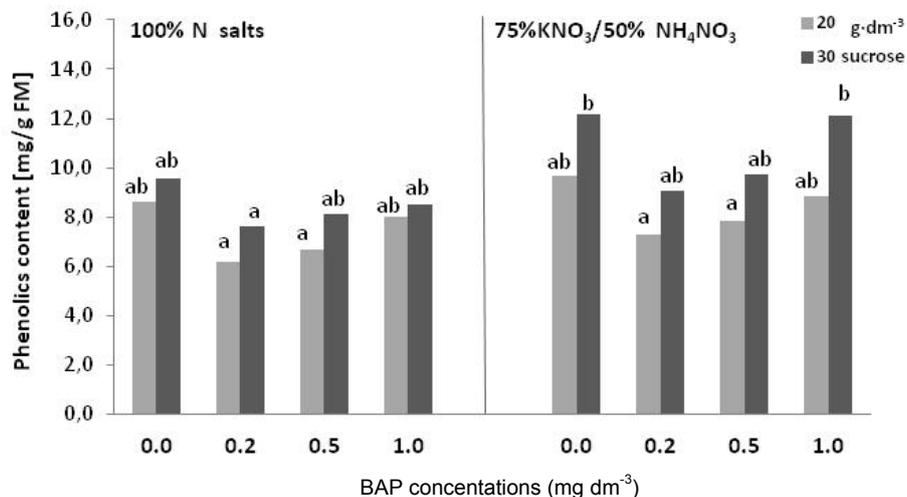


Fig. 4. The effect of different nitrogen levels (in relation to MS medium) and BAP and sucrose concentrations on the total phenolics content in the shoots of *M. × soulangiana* 'Coates' after a 5-week subculture period. Means each growth parameter indicated with the same letter do not differ significantly ($\alpha = 0.05$) according to Duncan's test

tion (1.0 mg·dm⁻³) (fig. 4). The results of the study showed that the elevated levels of phenolics in the shoots of *M. × soulangiana* 'Coates' did not always coincide with decreased shoot formation (fig. 1, 4). Moreover, the study showed that the phenolic content in the shoots did not coincide with the leaf browning observed during shoot multiplication (fig. 2, 4).

DISCUSSION

The activation of axillary buds plays a main role in propagation of *Magnolia in vitro*. It is known that induction of shoot formation is significantly affected by cytokinins, but the effectiveness of multiplication depends on the number of factors. Previous studies had indicated that the basal salt composition and plant growth regulators were important factors influencing axillary multiplication of *Magnolia* sp. *in vitro* [Biedermann 1987, Kamenická and Lanakova 2000, Parris et al. 2012]. Depending on the genotype, the most effective shoot formation has been obtained on media with low or high ionic concentrations [Isac 1996, Gabryszewska 1997, Radomir 2012, Parris et al. 2012, Sokolov et al. 2014]. BAP was found the most effective in activation of axillary bud of various *Magnolia* species and cultivars. The optimal BAP concentration, however, was different according to the genotype and was ranging from 0.25 to 5.0 mg·dm⁻³ [Isac 1996, Gabryszewska 1997, Kamenická and Lanakova 2000, Parris et al. 2012, Radomir 2012, Sokolov et al. 2014]. The authors did not show the interaction of growth regulator and nutritional factors on the growth and development of *magnolia in vitro*.

There are several reports showing that sucrose and nitrogen salts had important influence on axillary bud activity in plants propagated *in vitro*, but the optimum sucrose-to-nitrogen salts ratio for axillary branching strongly depends on the plant species and genotype. In *Syringa vulgaris*, increased sucrose concentrations (5–30 g·dm⁻³) reduced the axillary shoot formation, but nitrogen salts supply overcame the inhibitory effect of sucrose [Gabryszewska 2011]. The shoot formation of *Clematis pitcheri* was stimulated by lowering the strength of nitrogen salts by half and sucrose to 10 g·dm⁻³ [Gabryszewska et al. 2008]. Similarly to *Camelia japonica* [Wojtania et al. 2011], the activity of axillary shoots of *M. × soulangiana* ‘Coates’ was reduced in the presence of sucrose in concentration of 30 g·dm⁻³. The results of present study showed, that sucrose-inhibition of magnolia shoot formation depended on sucrose-to-nitrogen salts ratio and cytokinin level. Current knowledge indicates that plants developed mechanism that allow them to sense and respond to changes in levels of carbon and nitrogen metabolites [Coruzzi and Zhou 2001, Zheng 2009]. Recent studies have provided increasing evidence of coordination between nutritional and hormonal signaling [Rubio et al. 2009]. It has been suggested that hormonal signaling pathway control the growth and development of plant in response to fluctuation of nutrient level [Krouk et al. 2011]. A number of studies have shown that the nitrogen availability modulated cytokinin biosynthesis, accumulation and translocation. Cytokinins function as local signal or long-distance signal that control nitrogen uptake and assimilation [Kiba et al. 2011]. Our study showed that the increased BAP concentration from 0.2 to 1.0 mg·dm⁻³ in the medium with reduced level of nitrogen salts, slightly stimulated or significantly reduced the shoot and leaf formation according to sucrose concentration (20 and 30 g·dm⁻³, respectively). On medium containing full strength nitrogen salts, increasing BAP concentration significantly reduced the shoot formation of *M. × soulangiana* ‘Coates’ at both levels of sucrose. There are few reports showing the effect of different cytokinin concentrations on magnolia shoot formation. An increase in shoot formation in *M. × soulangiana* ‘Alexandrina’ was observed when BAP concentration increased from 0.5 to 1.5 mg·dm⁻³ [Gabryszewska 1997]. In *Magnolia* ‘Ann’ increasing BAP level from 0.45 to 1.8 mg·dm⁻³ had no effect on multiplication rate [Parris et al. 2012]. For the first time, our research has shown that optimal BAP concentration for effective shoot formation of *Magnolia in vitro* depends on sucrose/nitrogen salts ratio.

In the literature, the tissue browning and subsequent death of cultured explants as well as a low multiplication rate of the same magnolia genotype have been reported to be caused by phenolic substances [Bidermann 1987, Kamenická et al. 1996], but their levels in magnolia shoots have not been quantify. Plant phenolics are secondary metabolites that encompass several structurally diverse classes of natural products arising from the shikimate-phenylpropanoids-flavonoids pathways [Boudet 2007]. It has been demonstrated that regulation of secondary metabolism is closely related to nitrogen and carbon metabolism [Fritz et al. 2006]. Phenylpropanoid metabolism can be induced by various stresses, low availability of nutrients or increased C/N ratio by supplying an external carbon source or limiting nitrogen level [Singh et al. 1998, Leser et al. 2002]. Our study showed that phenolics formation in *M. × soulangiana* shoots changed according to cytokinin, nitrogen and sucrose levels and their ratio. Similarly to *Malus × domestica* propagated *in vitro* [Lux-Endrich et al. 2000], the phenolics synthesis in ma-

gnolia shoots was enhanced under conditions of high sucrose-to-nitrogen salts ratio. Our study have shown that cytokinin modify phenolics production in *M. × soulangiana* shoots. BAP, in low concentration lowered phenolics level as compared to the control medium, however, increasing BAP concentration from 0.2 to 1.0 mg·dm⁻³ significantly enhanced total phenolics level. Cytokinin-stimulation of phenolics accumulation was observed in different plant species, including *Lens culinaris* [Giannakoula et al. 2012], *Tectona grandis* [Quiala et al. 2012] and *Strelizia reginae* [North et al. 2012]. The mechanism by which cytokinins induce or stimulate phenolics production is still unclear.

It has been reported that phenolics can inhibit or stimulate the growth and development of plants *in vitro* and their effect strongly depends on the genotype [Lorenzo et al. 2001, Ozyigit 2008, Palacio et al. 2012]. Variation in the activity of phenolics is explained by the variation in their structure and concentration as well as the variation in the conditions influencing their mode of action [Appel 1993]. Our study showed that increased phenolics level coincident with decreased shoot formation capacity when *M. × soulangiana* 'Coates' was grown on the medium with reduced level of nitrogen salts, high sucrose (40 g·dm⁻³) and increased BAP levels (0.2–1.0 mg·dm⁻³). Full strength nitrogen salts supply lowered sucrose- and cytokinin-dependent phenolics production, but it did not translate into enhanced shoot formation.

As shown by our study, the enhanced phenolics content in *M. × soulangiana* 'Coates' during shoot multiplication did not coincide with leaf browning. It has been observed that leaf browning was caused by unsuitable levels and balance of cytokinin, nitrogen salts and sucrose in the medium. It is known that leaf browning can be a symptom of shoot tip necrosis (STN), a common physiological disorder affecting a wide range of plant species [Sha et al. 1985]. One of the most important factors affecting STS is cytokinin. However, some researchers have observed increased STN after eliminating, reducing or increasing the concentration of cytokinin [Bairu et al. 2009]. Our findings indicate that enhanced BAP concentration can stimulate as well as to inhibit leaf browning in *M. × soulangiana* 'Coates' depending on the sucrose/nitrogen salts ratio in the MS medium. The highest leaf browning was observed on full strength MS medium, containing 20 g·dm⁻³ sucrose and 1.0 mg·dm⁻³ BAP.

The results reported here indicate that the low activity of axillary buds and leaf browning in *M. × soulangiana* 'Coates' is the result of unsuitable levels and balance between cytokinin, sucrose and nitrogen salts in the medium. Using the medium containing low BAP level (0.2 mg·dm⁻³), 20 g·dm⁻³ sucrose and reduced nitrogen salts level (in relation to MS medium) allowed us to obtain cyclic multiplication of different *M. × soulangiana* cultivas (data not shown).

CONCLUSION

1. Our results show that the growth and development of *M. × soulangiana* 'Coates' *in vitro* as well as phenolics production depend on the levels and ratio of cytokinin, sucrose and nitrogen salts in the medium.

2. The main factor inducing the axillary shoot formation is cytokinin (BAP).

3. Increasing BAP concentration (0.2–1.0 mg·dm⁻³) can stimulate or inhibit the shoot formation and phenolics production according to the sucrose/nitrogen salts ratio in the medium.

4. The enhanced phenolics level in magnolia shoots did not always coincide with decreased shoot formation.

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WPLYW RÓŻNEGO POZIOMU CYTOKININY, SACHAROZY I SOLI AZOTOWYCH NA WZROST I ROZWÓJ ORAZ POZIOM SUBSTANCJI FENOLOWYCH W PĘDACH *Magnolia × soulangiana* ‘Coates’ *in vitro*

Streszczenie. Substancje fenolowe wytwarzane podczas wzrostu magnolii *in vitro* są uważane za czynnik hamujący tworzenie się pędów. Celem badań było określenie wpływu i współdziałania różnego stężenia soli azotowych, sacharozy i cytokininy na wzrost i rozwój pędów *Magnolia × soulangiana* ‘Coates’ *in vitro* w zależności od poziomu substancji fenolowych. Badania wykazały, iż tworzenie zarówno pędów i liści, jak i substancji fenolowych, istotnie zależało od stężenia i wzajemnych proporcji cytokininy, sacharozy i soli azotu w pożywce Murashige and Skooga (MS). Najwyższy współczynnik mnożenia się pędów (4,8 pędów/eksplantat) uzyskano na pożywce zawierającej 0,2 mg·dm⁻³ benzyloaminopuryny (BAP), 100% soli azotowych wg MS i 20 g·dm⁻³ sacharozy. Przy tym poziomie sacharozy wzrost stężenia BAP (0,2–1,0 mg·dm⁻³) powodował silną inhibicję lub nieznaczną stymulację tworzenia się pędów w zależności od poziomu azotu, kolejno 100% i 75/50%. Przy niskim stosunku sacharozy do soli azotowych w pożywce, wzrost stężenia BAP indukował brązowienie liści. Czynnikiem istotnie hamującym tworzenie się pędów i liści magnolii *in vitro* była sacharoza zastosowana w stężeniu 30 g·dm⁻³, jednak jej działanie zależało od poziomu azotu i cytokininy. Przy wysokiej relacji sacharozy do soli azotowych, tworzenie się pędów było proporcjonalne do stężenia BAP. Wysoka relacja sacharozy do soli azotowych w pożywce wpływała również na podwyższony poziom substancji fenolowych w pędach. W porównaniu z kontrolą (bez cytokininy), pędy rosnące w obecności BAP produkowały mniej substancji fenolowych. Przy wysokim stosunku sacharozy do soli azotowych, wraz ze wzrostem stężenia cytokininy obserwowano jednak istotny wzrost ich poziomu. Na podstawie wyników badań wnioskuje się, że nie we wszystkich traktowaniach podwyższony poziom substancji fenolowych w pędach korelował z inhibicją tworzenia się pędów.

Słowa kluczowe: BAP, brązowienie liści, pędy kątowe, stosunek sacharoza/sole azotowe

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