



MORPHOLOGICAL AND BIOCHEMICAL RESPONSES TO GIBBERELIC ACID IN *MAGNOLIA* × 'SPECTRUM' IN VITRO

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The total soluble sugar content and antioxidant enzyme activities were studied for the first time during axillary shoot formation in *Magnolia* × 'Spectrum' in vitro in response to BAP (0.3 mg l⁻¹), different levels of gibberellic acid (GA₃; 0.0, 0.1, 0.5, 1.0 mg l⁻¹), sucrose (20 and 30 g l⁻¹) and nitrogen salts (KNO₃/NH₄NO₃; 100/100% and 75/50% relative to MS medium). Among various GA₃ and sucrose/nitrogen salts ratios, the most effective axillary multiplication (5.9 shoots/explant) and leaf formation (25.7 leaves per multiplied clumps) were obtained after addition of GA₃ at 0.1 mg l⁻¹ to a BAP medium containing 20 g l⁻¹ sucrose and reduced levels of nitrogen salts (75% KNO₃ and 50% NH₄NO₃). The addition of GA₃ to the BAP medium enhanced shoot formation by 36% and leaf formation by 27%. The highest shoot formation capacity of *M.* × 'Spectrum' in vitro coincided with enhanced levels of soluble sugar and peroxidase (POD) activity. Increasing GA₃ concentration from 0.1 to 1.0 mg l⁻¹ in the above medium resulted in inhibition of shoot and leaf formation and a decrease in the soluble sugar content. The influence of GA₃ on the activities of catalase (CAT) and POD depended on its concentration and the levels of sucrose and nitrogen salts in the medium. The highest increase in CAT and POD activities, that coincided with the enhanced shoot formation capacity of *M.* × 'Spectrum' in vitro, was observed after addition of GA₃ to the medium containing high levels of sucrose and nitrogen salts.

Keywords: antioxidant enzymes, axillary shoot formation, soluble sugar accumulation, sucrose to nitrogen salt ratio

INTRODUCTION

Magnolias (Magnoliaceae) have long been popular ornamental plants, recommended for planting in gardens and urban green areas. *Magnolia* × 'Spectrum' was bred in the US National Arboretum in 1963 and is a cross between *M. liliflora* 'Nigra' and *M. sprengeri* 'Diva'. It is a medium-sized tree with a conical habit, valuable for its very large, tulip-shaped, deep red-purple flowers appearing before the leaves, and for high resistance to frost. Due to difficulties in propagation of magnolias by cutting or grafting, development and optimization of in vitro propagation methods is desirable.

Magnolias are most often propagated by activation of axillary buds, which guarantees high genetic stability of the obtained plant material. It has been reported that obtaining high activity of axillary buds is not easy and depends on many factors, including the composition of basal salts, plant growth

regulators and the genotype (Biedermann, 1987; Kamenicka and Lanakova, 2000; Podwyszyńska et al., 2000; Parris et al., 2012; Sokolov et al., 2014). Our previous study showed that growth and development of *M.* × *soulangiana* 'Coates' in vitro was significantly influenced by the levels and ratio of cytokinin, sucrose and nitrogen salts in the MS medium (Wojtania et al., 2015). Among different magnolia cultivars grown in vitro, *M.* × 'Spectrum' is characterized by relatively low activity of axillary buds.

In many plant species, low activity of axillary buds is caused by the paradormancy phenomenon, also known as apical dominance or correlative inhibition. It is known that bud activity is regulated by various factors, including phytohormones, nutrients and temperature (Chae et al., 2006). Shoot branching is highly stimulated by cytokinin. However, sometimes cytokinin is not sufficient for bud outgrowth because other factors affect the competency

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for buds to respond to cytokinin (Dun et al., 2012). Other factors that are involved in the regulation of plant and bud dormancy are gibberellins (GAs) and sugars (Horvath et al., 2002; Chae et al., 2006). A positive effect of gibberellic acid (GA_3) on bud break has been observed in some woody plant species cultured in vitro, including *Camelia japonica* (Beretta and Eccher, 1987; Wojtania et al., 2011) and *Vitex negundo* (Sahoo and Chand, 1998). On the other hand, many genotypes responded negatively to exogenous GA application. For example, GA_3 added to a cytokinin-medium inhibited shoot formation in *Ricinus communis* (Sujatha and Reddy, 1998), and induced leaf chlorosis and bud necrosis in *Ficus carica* (Fraguas et al., 2004). To our knowledge, there is no information on the influence of GA_3 on axillary bud activity in magnolia.

It is believed that enhanced regeneration potential and shoot formation capacity correlate with increased activity of antioxidant enzymes (Mitrović et al., 2012). Plant hormones are known to be involved in antioxidant responses of many plants (Mýtinová et al., 2011). For example, an influence of exogenously applied cytokinin on catalase (CAT) and peroxidase (POD) activities has been observed in axillary multiplication of *Pelargonium hortorum* (Wojtania and Skrzypek, 2014), and adventitious shoot formation in *Pinus strobus* (Tang and Newton, 2005). So far there has been no information on the effect of GA_3 on antioxidant enzyme activities in relation to magnolia shoot formation.

The present study was undertaken to clarify the role of GA_3 in formation of shoots in *Magnolia* × ‘Spectrum’ in vitro. Morphological and biochemical responses of magnolia shoots grown in the presence of different concentration of GA_3 , sucrose and nitrogen salts were studied.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Shoot cultures of *Magnolia* × ‘Spectrum’ were initiated from apical and axillary bud explants, collected from two-year-old field-grown plants. The initial explants and the subsequent subcultures of axillary shoots were performed on Murashige and Skoog (1962) basal medium containing 100 mg l⁻¹ myo-inositol, nicotinic acid, pyridoxine and thiamine (1.0 mg l⁻¹ each), 1.0–1.5 mg l⁻¹ benzylaminopurine (BAP), 30 g l⁻¹ sucrose and solidified with 6.5 g l⁻¹ LAB-AGAR (Biocorp Poland). The pH of the medium was adjusted to 5.6 before autoclaving. The culture of axillary shoots was subcultured on a fresh medium every 5 weeks. After a few subcultures the shoots showed progressive hyperhydricity, as well as inhibited activity of axillary buds.

SHOOT GROWTH AND DEVELOPMENT

Single shoots harvested from shoot cultures were used as explants. The magnolia shoots were cultured on MS medium containing benzylaminopurine (BAP; 0.3 mg l⁻¹) and different concentration of gibberellic acid (GA_3 ; 0.0, 0.1, 0.5, 1.0 mg l⁻¹), nitrogen salts (KNO_3/NH_4NO_3 ; 100/100% and 75/50% relative to the MS medium), and sucrose (20 and 30 g l⁻¹). The concentration of cytokinin was determined on the basis of previous experiments (Wojtania et al., 2015). Explants grown on a medium without GA_3 were used as controls.

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. Each treatment consisted of 25 explants. The experiment was repeated twice. The observations and measurements were recorded after 2 subcultures on the same medium. The number and length of the axillary shoots, the number of leaves and the number of brown leaves (%) were determined. Leaf samples were also collected at that time for determination of the soluble sugar content as well as peroxidase (POD) and catalase (CAT) activities.

ANALYSIS OF SOLUBLE SUGAR

Soluble sugars in the leaves after homogenization were extracted with 1 ml of 80% aqueous ethanol, then centrifuged at 2800 rpm for 10 min. The amount of total sugars was estimated by the phenol-sulfuric method (Dubois et al., 1956). The supernatant was mixed with 5% phenol and sulphuric acid. The absorbance (λ=490 nm) of the samples was measured spectrophotometrically on a micro-plate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). The amounts of soluble sugars were expressed in milligrams (mg) per 1 g of fresh mass (FM) of plant tissue.

ACTIVITY OF ANTIOXIDATIVE ENZYMES

Plant tissue was homogenized at 4°C with a phosphate buffer (pH 7.8) containing 0.01 M EDTA and 0.5% BSA. The homogenate was centrifuged at 2800 rpm for 10 min.

The activity of catalase (CAT, EC 1.11.1.6) was measured spectrophotometrically (λ=240 nm) by the modified method of Aebi (1984). The reaction mixture consisted of 200 μl 0.05 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 50 μl of 0.03 M H₂O₂ in this buffer and 5 μl of the supernatant.

The activity of peroxidase (POD, EC 1.11.1.11) was measured by the modified method of Lück (1962). The measurement was carried out spectrophotometrically (λ=460 nm), by measuring in 300 μl of 0.05 M phosphate buffer containing

0.1 mM EDTA, pH 7.0, the amount of the products of 5 µl of 1% p-phenylenediamine and 5 µl supernatant. The reaction was started in the presence of 5 µl of 0.03 M H₂O₂.

The reaction kinetics for all the enzymes was examined spectrophotometrically on a microplate reader (Synergy 2, Bio-Tek, Winooski, VT, USA) after 60 and 120 s since the initiation of the reaction.

The data were subjected to a three factor analysis of variance in relation to exogenous GA₃, nitrogen and sucrose treatments. The means were compared by Duncan's test at the α=0.05 significance level.

RESULTS

SHOOT GROWTH AND DEVELOPMENT

The results of our study showed that the addition of GA₃ to a BAP medium increased shoot and leaf formation in *Magnolia* × 'Spectrum' in vitro, but its

effect on those processes significantly depended on the sucrose/nitrogen salt ratio in the MS medium (Fig. 1a and 2a). The most effective axillary multiplication and leaf formation was observed on the medium containing 20 g l⁻¹ sucrose and reduced levels of nitrogen salts (75% KNO₃ and 50% NH₄NO₃ relative to the MS medium). On this medium, GA₃ applied together with BAP enhanced shoot formation in *M. × 'Spectrum'* by 36% and leaf formation by 27%. The highest multiplication rate (5.9 shoots/explants) and the highest number of leaves (25.7 leaves per multiplied clumps) were obtained in the presence of 0.1 mg l⁻¹ GA₃. Increasing GA concentration from 0.1 to 1.0 mg l⁻¹ resulted in inhibition of shoot and leaf formation (Fig. 1a, 2a and 3). Lateral bud outgrowth was completely suppressed at 1.5 mg l⁻¹ GA₃ (data not shown).

The development of *M. × 'Spectrum'* axillary shoots and leaves was significantly inhibited by sucrose at a concentration of 30 g l⁻¹ (Fig. 1a and 2a). The sucrose-inhibition of shoot forma-

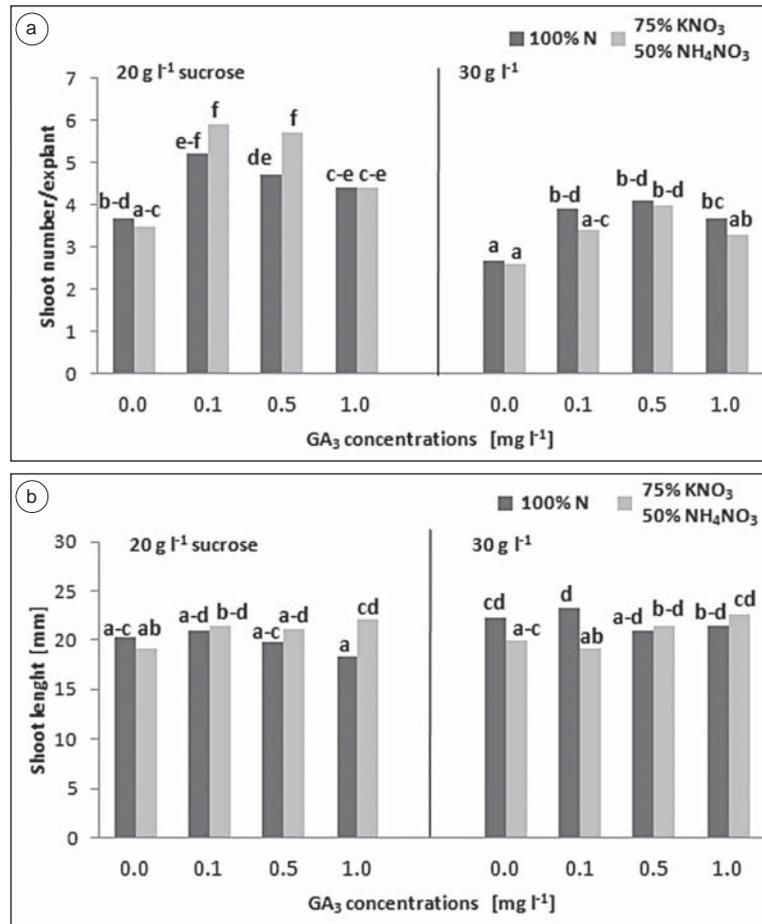


Fig. 1. Effect of GA₃, added to MS medium containing BAP (0.3 mg l⁻¹), different concentrations of sucrose and nitrogen salts on shoot formation (a) and shoot length (b) in *M. × 'Spectrum'* after a 5-week subculture period. Means of each growth parameter designated with the same letter do not differ significantly (α=0.05) according to Duncan's test.

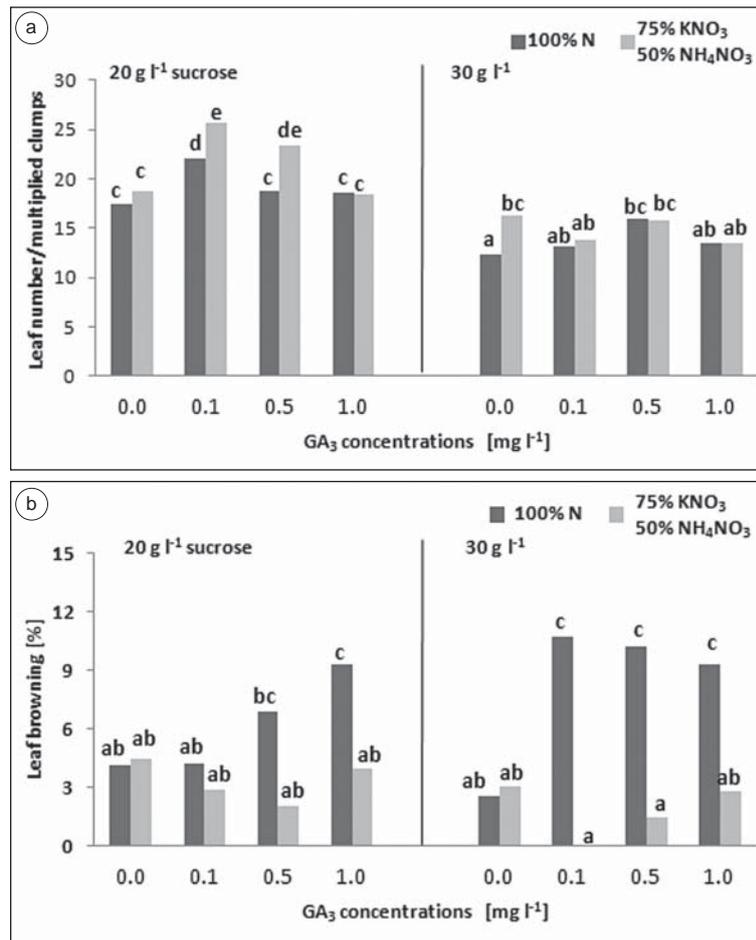


Fig. 2. Effect of GA₃, added to MS medium containing BAP (0.3 mg l⁻¹), different concentrations of sucrose and nitrogen salts on leaf formation (a) and leaf browning (b) in *M. × 'Spectrum'* after a 5-week subculture period. Means of each growth parameter designated with the same letter do not differ significantly ($\alpha=0.05$) according to Duncan's test.



Fig. 3. Shoots of *M. × 'Spectrum'* after a 5-week subculture period on media containing different concentrations of GA₃, 0.3 mg l⁻¹ BAP, 20 g l⁻¹ sucrose and reduced levels of nitrogen salts (KNO₃/NH₄NO₃: 75/50%).

tion was partly overcome by exogenous GA₃. However, the highest multiplication rate (4.1 shoots/explants) obtained in the presence of BAP and GA₃ (0.5 mg l⁻¹) was decidedly lower than the multiplication rate of the shoots growing on the medium containing 20 g l⁻¹ sucrose (Fig. 1a). In the presence of 30 g l⁻¹ sucrose, the GA-stimulation of shoot formation was greater on the medium containing full strength MS nitrogen salts. In general, the application of GA₃ did not overcome the sucrose-inhibition of leaf formation in *M. × 'Spectrum'* plantlets, with the exception of the shoots growing in the presence of 0.5 mg l⁻¹ GA₃ and reduced levels of nitrogen salts (Fig. 2a). As shown in Fig. 2b, the addition of GA₃ to the BAP medium containing full strength nitrogen salts induced leaf browning in *M. × 'Spectrum'* shoots, but its effect depended on the concentration and sucrose level. In the presence of 20 g l⁻¹ sucrose, the leaf browning was directly proportional to GA₃ concentration. On the medium containing 30 g l⁻¹, however, the rapid increase of leaf browning was observed at the lowest GA₃ level (0.1 mg l⁻¹). The GA-induction of leaf browning was the greatest on the medium without cytokinin (data not shown).

SOLUBLE SUGAR CONTENT

The soluble sugar content in *M. × 'Spectrum'* leaves was measured after five weeks of growth on media containing BAP and different concentrations of GA₃, sucrose and nitrogen salts in the MS medium. On the control medium (without GA₃), the soluble sugar content in the shoots was signifi-

cantly influenced by high sucrose-to-nitrogen salt ratio (Fig. 4). On the medium with reduced level of nitrogen salts, increasing exogenous sucrose supply (from 20 to 30 g l⁻¹) resulted in 36% increase of soluble sugar content in magnolia shoots. The addition of GA₃ to the BAP medium significantly affected the sugar level in magnolia shoots, but the GA₃-mediated effect depended on its concentration and the sucrose/nitrogen salts ratio in the medium (Fig. 4). There was the highest increase in the endogenous sugar level (by 48% relative to the control) by the applied GA₃ at 0.1 mg l⁻¹ to the medium enriched with BAP, 20 g l⁻¹ sucrose and reduced levels of nitrogen salts (75% KNO₃ and 50% NH₄NO₃ relative to the MS medium). However, increasing GA₃ supply (from 0.1 to 1.0 mg l⁻¹) lowered the sugar content in magnolias growing on the medium with the reduced nitrogen salts level (irrespective of sucrose concentration in the medium). On the medium with full strength MS nitrogen salts, the addition of 0.1 mg l⁻¹ GA₃ together with BAP had no significant effect on the sugar content relative to the control, but increasing GA₃ supply (from 0.1 to 1.0 mg l⁻¹) enhanced the sugar content in magnolia shoots grown both in the presence of 20 g l⁻¹ and 30 g l⁻¹ sucrose (Fig. 4).

ACTIVITY OF ANTIOXIDATIVE ENZYMES

The activities of antioxidant enzymes in *M. × 'Spectrum'* leaves were measured after a five-week subculture period. On the control medium containing BAP only, CAT and POD activities were enhanced by lowering the concentration of nitro-

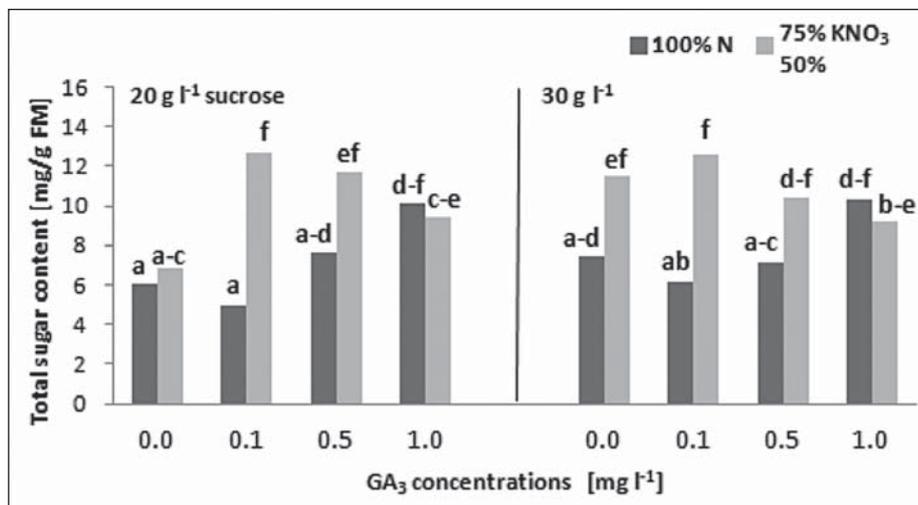


Fig. 4. Effect of GA₃, added to MS medium containing BAP (0.3 mg l⁻¹), different concentrations of sucrose and nitrogen salts on the total soluble sugar content in *M. × 'Spectrum'* after a 5-week subculture period. Means designated with the same letter do not differ significantly ($\alpha=0.05$) according to Duncan's test.

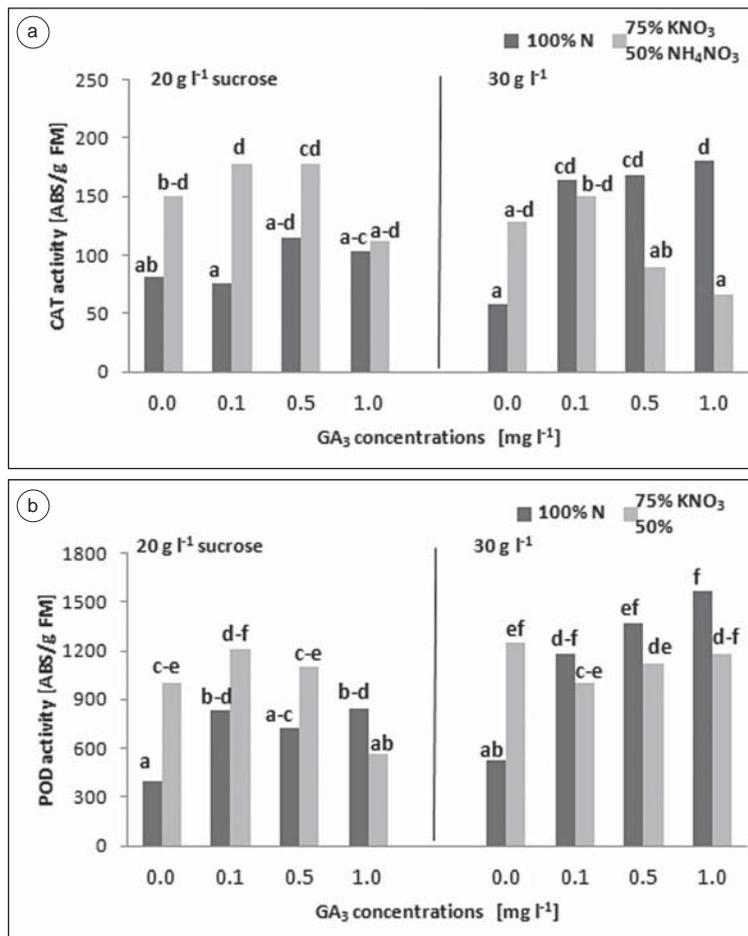


Fig. 5. Effect of GA₃, added to MS medium containing BAP (0.3 mg l⁻¹), different concentrations of sucrose and nitrogen salts on the catalase (a) and peroxidase (b) activities in *M. × 'Spectrum'* after a 5-week subculture period. Means of each growth parameter designated with the same letter do not differ significantly ($\alpha=0.05$) according to Duncan's test.

gen salts in the MS medium (Fig. 5a and 5b). Irrespective of the sucrose levels, the reduced level of nitrogen salts (75% KNO₃ and 50% NH₄NO₃) enhanced CAT activity by 50% and POD activity by 60% relative to the medium containing 100% MS nitrogen salt levels. The application of GA₃ together with BAP significantly affected the antioxidant enzyme activities in *M. × 'Spectrum'* shoots, but the effect of GA₃ depended on its concentration and the levels of sucrose and nitrogen salts ratio in the medium (Fig. 5a and 5b). On the medium supplemented with 20 g l⁻¹ sucrose, GA₃ had not significant influence on CAT activities, irrespective of the nitrogen levels. On the medium containing 30 g l⁻¹ sucrose, GA₃ (regardless of concentration) significantly enhanced (by 65%) CAT activities in the presence of full strength MS nitrogen salts. On the medium with reduced levels of nitrogen salts, increasing GA₃ concentration from 0.1 to 1.0 mg l⁻¹

decreased CAT activities, but the differences were not statistically important (Fig. 5a). As shown in Fig. 5b, the highest increase in POD activity (by 70%) was stimulated by GA₃ (in a concentration-dependent manner) on the medium containing high levels of sucrose and nitrogen salts. The promoting effect of GA₃ on CAT and POD activities on the medium containing high levels of sucrose and nitrogen salts coincided with the enhanced shoot formation capacity of *M. × 'Spectrum'* in vitro. In the presence of 20 g l⁻¹ sucrose, significant increase in activity of POD was observed when 0.1 mg l⁻¹ GA₃ was added to the medium with reduced levels of nitrogen salts and 0.1 and 1.0 mg l⁻¹ GA₃ to the medium containing full strength MS nitrogen salts. Enhanced POD activity observed on the medium with the reduced levels of sucrose and nitrogen salts coincided with the highest rate of shoot formation in *M. × 'Spectrum'* in vitro.

DISCUSSION

Gibberellins (GAs) play a key role in the control of cell division, elongation and the correlative inhibition phenomenon (Horvath et al., 2002; Zhang et al., 2008). In plants propagated in vitro, addition of GA₃ to the medium has been found to stimulate (Beretta and Eccher, 1987; Gabryszewska, 2009; Wojtania et al., 2011) or inhibit (Sujatha and Reddy, 1998; Fraguas et al., 2004) lateral bud outgrowth. The increased rate of shoot formation was usually a result of a combined treatment with GA₃, cytokinin and sucrose (Gabryszewska, 2009, 2015; Wojtania et al., 2011). Like in other plant species, the shoots of *M. × 'Spectrum'* treated with GA₃ only had no capacity for shoot formation, and additionally leaf browning was observed (data not shown). In the case of magnolia, the highest rate of shoot formation was found on the medium containing a low level of GA₃ (0.1 mg l⁻¹). However, the optimal GA₃ level for increasing axillary multiplication was found to vary between genotypes. For example, it was 10 mg l⁻¹ in *Paeonia lactiflora* (Gabryszewska, 2009), 0.7-1.0 mg l⁻¹ in *Camelia japonica* (Beretta and Eccher, 1987; Wojtania et al., 2011) and 0.1-0.5 mg l⁻¹ in various wild rose species (Pawłowska, 2011).

The results presented in this paper demonstrate that both the morphological and biochemical responses to GA₃ significantly depended on the levels of sucrose and nitrogen salts in the medium. The amounts of sucrose and nitrogen salts and their ratio in the media were found to be an important factor regulating formation of shoots of different plant species propagated in vitro, including *Syringa vulgaris* and *Helleborus niger* (Gabryszewska, 2011, 2015). The high concentration of sucrose inhibited the outgrowth of axillary buds and development of *Peonia lactiflora* propagated in vitro but exogenous gibberellins can overcome the sugar-induced inhibition and stimulate shoot formation and growth in the presence of cytokinins (Gabryszewska, 2009). In the case of *M. × 'Spectrum'*, however, GA₃ partly overcame the sucrose-induced inhibition of the shoot formation but not the leaf formation.

The present study indicates that *Magnolia* shoots treated with GA₃ contain more soluble sugar and show higher activity of antioxidant enzymes, relative to the control. It has been reported that GA treatments led to an increase in sugar content in different plant species, including *Phalaenopsis amabilis* and *Rosa hybrida* that correlated with floral initiation (Chen et al., 1994) and shoot branching (Choubane et al., 2012). In *M. × 'Spectrum'*, the highest increase in the soluble sugar content was observed when 0.1 mg l⁻¹ GA₃ was added to the BAP medium with 20 g l⁻¹ sucrose and reduced lev-

els of nitrogen salts (75% KNO₃ and 50% NH₄NO₃ relative to the MS medium). In this case, the enhanced sugar content coincided with the highest rate of shoot formation. On the other hand, increased sugar content was also observed in the shoots growing on GA₃-free medium supplemented with BAP, 30 g l⁻¹ sucrose and reduced amounts of nitrogen salts. It could be related to the accumulation of different soluble sugars in *M. × 'Spectrum'* shoots. The available evidence suggests that some processes may be mediated by the absolute levels of a particular sugar, such as glucose or sucrose, whereas other ones by flux through particular sugars (Gibson, 2003). A relationship between sugar availability and bud outgrowth has been reported for different plant species, including *Rosa hybrida* (Barbier et al., 2015). In this process, sucrose was suggested not only to play a trophic role, but also to act as a signaling molecule (Barbier et al., 2015).

High levels of both sugar and nitrogen have been found to be inhibitory to GAs production and responsiveness (Candau et al., 1992; Perata et al., 1997). It has been demonstrated that many magnolia genotypes prefer media containing a lower nitrogen concentration than that of MS medium (Biedermann, 1987; Kamenicka and Lanakova, 2000; Wojtania et al., 2015). It is believed that magnolia shoots growing on media containing low levels of sucrose and/or low levels of nitrogen salts may contain higher levels of endogenous GAs required for enhanced shoot formation in magnolia in vitro. Accumulation of GAs in vegetative buds has been shown to promote the shift from meristematic identity to organ differentiation (Dodsworth, 2009).

GAs are also known to alter plant metabolism under stress conditions (Iqbal et al., 2011). In some plant species, including *Cucumis sativus* and *Lycopersicon esculentum*, it has been shown that the GA₃-improved stress tolerance correlates with enhanced activity of antioxidant enzymes (Li et al., 2011; Khavari-Nejad et al., 2013). In other plants, such as *Vigna radiata* and *Zea mays*, GA₃ treatments reduced the activities of antioxidant enzymes (Chakrabarti and Mukharji, 2003; Tuna et al., 2008). As shown in our study, the addition of GA₃ to the BAP medium stimulated or inhibited CAT and POD activities in magnolia shoots depending on the levels of sucrose and nitrogen salts in the medium. The highest increase in CAT and POD activities was observed after the addition of GA₃ to the medium containing high levels of sucrose and nitrogen salts.

The results presented here showed the importance of interaction between GA₃, sucrose and nitrogen in the morphological and biochemical responses of *M. × 'Spectrum'* plantlets in vitro. For the first time, it has been found that GA₃-enhanced shoot formation in magnolia in vitro coincided with

enhanced content of soluble sugar and antioxidant enzyme activities. It is suggested that the shoot formation response to GA₃ may be related to accumulation of a specific kind of sugar, but it needs further study. The improved shoot formation capacity obtained after the addition of GA₃ at 0.1 mg l⁻¹ and 0.5 mg l⁻¹ to a BAP medium containing 20 g l⁻¹ sucrose and reduced levels of nitrogen salts (75% KNO₃ and 50% NH₄NO₃) can be very useful for mass propagation of *M. × 'Spectrum'* in vitro.

AUTHORS' CONTRIBUTIONS

AW – designed and performed the experiments, analyzed data and wrote the paper; ES – was responsible for the plant tissue analysis; EG – critically read and revised the manuscript. The authors declare that there are no conflicts of interest.

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