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ORIGINAL ARTICLE

Growth, secondary metabolite production and antioxidant enzyme response of *Morinda citrifolia* adventitious root as affected by auxin and cytokinin

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Abstract *Morinda citrifolia* adventitious roots were cultured in shake flasks using Murashige and Skoog medium with different types and concentrations of auxin and cytokinin. Root (fresh weight and dry weight) accumulation was enhanced at 5 mg l^{-1} indole butyric acid (IBA) and at 7 and 9 mg l^{-1} naphthalene acetic acid (NAA). On the other hand, 9 mg l^{-1} NAA decreased the anthraquinone, phenolic and flavonoid contents more severely than 9 mg l^{-1} IBA. When adventitious roots were treated with kinetin (0.1, 0.3 and 0.5 mg l^{-1}) and thidiazuron (TDZ; 0.1, 0.3 and 0.5 mg l^{-1}) in combination with 5 mg l^{-1} IBA, fresh weight and dry weight decreased but secondary metabolite content increased. The secondary metabolite content (including 1,1-diphenyl-2-picrylhydrazyl activity) increased more in TDZ-treated than in kinetin-treated roots. Antioxidative enzymes such as catalase (CAT) and guaiacol peroxidase (G-POD), which play important roles in plant defense, also increased. A strong decrease in ascorbate peroxidase activity resulted in a high accumulation of hydrogen peroxide. This indicates that adventitious roots can grow under stress conditions with induced CAT and G-POD activities and higher accumulations of secondary metabolites. These results suggest that 5 mg l^{-1} IBA supplementation is useful for growth and secondary metabolite production in adventitious roots of *M. citrifolia*.

Keywords Adventitious root cultures · Anthraquinone · Antioxidant · Biomass · *Morinda citrifolia* · Plant growth regulators

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Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EDTA	Ethylenediamine tetra-acetic acid
G-POD	Guaiacol peroxidase
IBA	Indole butyric acid
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
PGR	Plant growth regulator
PPF	Photosynthetic photon flux
ROS	Reactive oxygen species
TDZ	Thidiazuron

Introduction

Morinda citrifolia L. belongs to the Rubiaceae family, commonly known as Noni. It is native to Southeast Asia but has been extensively cultivated throughout the Indian subcontinent, Pacific islands, French Polynesia, Costa Rica, and Puerto Rico. It grows in relatively dry to moderately wet conditions, and at altitudes from sea level to about 1500 feet. It can be found near the coast, in lowlands, in grasslands or in gulches. It tolerates soil salinity and brackish irrigation water. Various parts of the plant, including its leaves, fruit, bark and roots, have been used for over 2000 years to treat several diseases such as high blood pressure and diabetes, and to cure eye problems, skin wounds, throat problems, respiratory ailments, constipation, and stomach pains (Wang et al. 2002). About 160 valuable phytochemicals have been identified in this plant, and the major compounds are polyphenolics, organic acids

and alkaloids (Wang and Su 2001). Among the phenolic compounds, the mostly commonly reported ones are anthraquinones. Cell culture is almost universally used in laboratories worldwide for the commercial production of valuable phytochemicals (Yu et al. 2005; Ali et al. 2005a). The effects of nutritional and physical factors on the formation of anthraquinone have been studied in suspension cultures of several plants, including *M. citrifolia* (Abdullah et al. 1998; Ahmed et al. 2008). However, low contents of anthraquinones and continuous foaming in the bioreactor during suspension cultures of *M. citrifolia* present major obstacles to the large-scale production of anthraquinones using *M. citrifolia* (Ahmed et al. 2008; Abdullah et al. 2000). In contrast, we performed a continuous study in which we observed that adventitious root cultures of *M. citrifolia* using large-scale bioreactor technology can be regarded as a promising method that overcomes the abovementioned problems (unpublished results).

Plant growth regulators are one of the most important factors affecting cell growth, differentiation and metabolite formation (Liang et al. 1991). The appropriate concentration of the medium is one of the critical determinants in controlling root growth and metabolite production. To produce cell dry mass as well as secondary metabolites from medicinal plants, it is important to establish the optimal culture conditions (chemical and physical environments) for the plant species used. The individual levels of auxin and cytokinin in the media used influence the growth and regulation of cell metabolism.

The exposure of plants to unfavorable conditions leads to the generation of reactive oxygen species (ROS) (Neil et al. 2002a). In general, oxidative stress results from the breakdown of cellular homeostasis of ROS production due to the excitation of molecular O_2 to form singlet oxygen and subsequently superoxide $^1O_2$ and hydrogen peroxide (H_2O_2). To counteract the toxicity of ROS, plants have developed defense systems that scavenge cellular ROS, enabling oxidative stress to be dealt with by nonenzymatic and enzymatic systems (Neil et al. 2002b). Such enzymes include catalase (CAT), guaiacol peroxidase (G-POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Zimmermann and Zentgraf 2005).

No reports are available on the growth, secondary metabolite production and antioxidant enzyme activities of adventitious root cultures of *M. citrifolia*. Therefore, the objective of this study was to optimize growth hormone concentrations for the growth of, the production of secondary metabolites by, and the antioxidant enzyme defense system of *M. citrifolia.* by trialing different concentrations of auxin and cytokinin or in combination with thidiazuron (TDZ) during adventitious root cultures of *M. citrifolia.*

Materials and methods

Plant material

Adventitious roots were induced from in-vitro-grown plantlets of *M. citrifolia* (Baque et al. 2009). The plantlets were grown from mature seeds after sterilizing the seeds with a 4% sodium hypochlorite solution for 20 min. The seeds were then soaked in a 2% sodium hypochlorite solution for 10 min under a laminar hood. Next, the seeds were washed in sterile distilled water until the sterilizer was completely removed and clipped using a sterilized clipper. Finally, the seeds were inoculated into test tubes containing 10 ml Murashige and Skoog (MS) medium without growth regulator.

Induction and proliferation of adventitious roots

The selected leaf explants (from in-vitro-grown plantlets) were placed on solid MS media supplemented with 1 mg l^{-1} indole butyric acid (IBA), 3% sucrose and 2.3% gelrite in a Petri dish containing 25 ml medium for adventitious root induction. Cultures were maintained under a 16 h photoperiod and a photosynthetic photon flux (PPF) of 20 μmol m^{-2} s^{-1} at 23 ± 2°C for 5 weeks. The induced adventitious roots were further proliferated in liquid MS media supplemented with 5 mg l^{-1} IBA, 3% sucrose and 10 g l^{-1} inoculum. The cultures were agitated at 100 rpm on a gyratory shaker in darkness at 23 ± 2°C for 4 weeks. The roots were subcultured every 4 weeks.

Treatment procedure

Effects of type and concentration of auxin on growth of adventitious root culture and production of secondary metabolites

Adventitious roots were grown under different concentrations of IBA and naphthalene acetic acid (NAA) (1, 3, 5, 7 and 9 mg l^{-1}) in full-strength MS medium containing 3% sucrose and 10 g l^{-1} inoculum in 250 ml Erlenmeyer flasks containing 100 ml liquid medium for 4 weeks. Roots were also grown in a hormone-free medium that served as the control. Root biomass and the anthraquinone, phenolic and flavonoid contents were analyzed after 4 weeks of culture. In combined treatment, the roots were grown under the abovementioned conditions after adding 5 mg l^{-1} IBA. Roots that were grown in a medium containing only 5 mg l^{-1} IBA served as the control. Root biomass and the anthraquinone, phenolic and flavonoid contents were analyzed after 4 weeks of culture. After the treatment, the roots were washed with double-distilled water to remove

medium from the root surface, blotted dry and used to check the respective parameters.

Sample collection for enzyme assay

Roots were collected after harvest for the enzyme assay and washed immediately with double-distilled water. One gram of roots was weighed into an Eppendorf tube and placed in liquid nitrogen, where it was stored at −80°C until further analysis.

Determination of anthraquinone, total phenolics, flavonoids and DPPH activity in roots

Roots were collected after each treatment and washed with double-distilled water and dried at 48°C for 3–4 days. The dried root sample (0.2 g) was digested in 40 ml 80% ethanol using a cod oil bath digestion system (LS-2050-S10, LS Tech, Korea) for 2 h at 80°C and filtered through filter paper (110 mm, Advantic, Japan). The resultant ethanolic extract was adjusted to 50 ml by adding more 80% ethanol. Anthraquinone was determined spectrophotometrically according to Zenk et al. (1975). The contents of total phenolics and flavonoids were analyzed spectrophotometrically from the same root ethanolic extract according to Wu et al. (2006). The antioxidant activity of each extract was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Hatano et al. (1988) with some modifications. 0.625 ml of DPPH (200 μM DPPH radical solution in 99.9% ethanol) solution were mixed with 0.375 ml of extract, vortexed and then incubated for 10 min at room temperature. The control was created by mixing 0.625 ml of DPPH with 0.375 ml 40% ethanol, and the absorbance was read against a blank at 517 nm.

Determination of the H_2O_2 level

Hydrogen peroxide levels were determined using a UV–visible spectrophotometer according to the method of Sergiev et al. (1997).

Enzyme extraction and protein estimation

One gram of fresh root tissues was ground in liquid nitrogen in a chilled pestle and mortar, and then homogenized in 2.0 ml 50 mM potassium phosphate buffer (pH 7.0) containing 2% (w/v) insoluble polyvinylpolypyrrolidone (PVP), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA. The homogenate was filtered through two layers of muslin cloth and centrifuged at 10000×*g* for 10 min at 4°C. To measure the APX activity, 5 mM ascorbic acid (AsA) was added to the extraction buffer. The soluble protein content was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay of enzymes

Catalase (EC 1.11.1.6) activity was determined in a reaction mixture containing 100 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.0) and enzyme extract. The disappearance of H_2O_2 after 10 min of reaction time was measured at 240 nm in both the blanks and the samples (modified after Bisht et al. 1989). For the blanks, 1 ml 2 N H_2SO_4 was added to the reaction mixture prior to the addition of enzyme extract. Guaiacol peroxidase (G-POD) activity was measured by monitoring the formation of tetraguaiacol (extinction coefficient of 6.39 mM^{-1} cm^{-1}) at 436 nm according to the method of Putter (1974). The ascorbate peroxidase (EC 1.11.1.11) activity was measured based on the method of Nakano and Asada (1981). APX and G-POD activities were expressed in terms of units (μmol min^{-1}) per milligram of protein.

Results and discussion

Auxin type and concentration

In order to determine the optimum concentrations of plant growth regulators, the adventitious roots were grown under different concentrations of IBA and NAA for 4 weeks (Table 1). The fresh weight and dry weight of the roots increased with increasing concentrations of both IBA (up to 5 mg l^{-1}) and NAA, and IBA was found to be more efficient at increasing the fresh weight and dry weight compared to NAA. The maximum fresh weight (54.02 g l^{-1}) and dry weight (4.48 g l^{-1}) were achieved at 5 mg l^{-1} IBA; the control fresh weight was 6.44 g l^{-1} and the control dry weight was 0.72 g l^{-1}. Previous investigators have suggested that the response of adventitious root growth to growth regulators varies from species to species. In the case of *Gymnema* cells, NAA was found to be more effective than IBA (Lee et al. 2006). The highest dry mass production from adventitious roots of *Echinacea angustifolia* was achieved at 2 mg l^{-1} IBA when compared to NAA (Wu et al. 2006). In adventitious root cultures of *Panax ginseng*, IBA was found to be more effective than NAA at promoting dry mass production (Kim et al. 2003). In *Karwinskia* root cultures, the highest dry root mass was produced under light on media supplemented with IBA when compared to NAA (Kollarova et al. 2004). They concluded that media enriched with NAA support the formation of callus-like mass bodies with very short roots, resulting in a lower dry mass. On the other hand, IBA

seems to be most effective substance for inducing and elongating roots in *Karwinskia* root cultures. To continue our optimization, we also checked anthraquinone, phenolic and flavonoid production under the same conditions. The maximum contents of anthraquinone (63.10 for IBA, 55.60 for NAA mg g^{-1} DW), phenolics (29.46 for IBA, 22.97 for NAA mg g^{-1} DW) and flavonoids (60.23 for IBA, 49.47 for NAA mg g^{-1} DW) were observed in roots cultured at 1 mg l^{-1} IBA and NAA, respectively (Table 2). High auxin levels are often deleterious to secondary metabolite accumulation (Chan et al. 2005). Higher concentrations of NAA (7 and 9 mg l^{-1}) decreased the secondary metabolite contents more than those of IBA did in our study. This clearly shows that the effect of auxin on secondary product formation markedly depends on the auxin types used and their concentrations. For example, in the case of the *M. citrifolia* cell culture, high concentrations of NAA reduced the production of anthraquinone (Zenk et al. 1975). On the other hand, the production of anthraquinone was induced by NAA and inhibited by 2,4-D in the same species (Hagendoorn et al. 1994). They observed that, in cultures treated with high concentrations of NAA, concomitant alkalization occurred in the cytoplasm, leading to cell death and a subsequent drop in metabolite accumulation. However, 5 mg l^{-1} NAA enhanced the secondary metabolite accumulation compared to a similar concentration of IBA in our study. These results clearly suggest that secondary product formation can be separated from growth. Based on the growth of our adventitious roots in terms of dry weight and production of metabolites, we selected 5 mg l^{-1} IBA as a suitable concentration for further experiments.

Table 1 Effects of the type and concentration of auxin on the adventitious root growth of *Morinda citrifolia* after 4 weeks

Treatments	Concentration (mg l^{-1})	Fresh weight (g l^{-1})	Dry weight (g l^{-1})
Control[a]	0	6.44 ± 0.58	0.72 ± 0.04
IBA	1	12.36 ± 0.30	1.44 ± 0.05
	3	36.62 ± 0.50	3.44 ± 0.19
	5	54.02 ± 1.23	4.84 ± 0.15
	7	53.40 ± 1.87	4.34 ± 0.09
	9	47.42 ± 1.43	4.18 ± 0.11
NAA	1	17.86 ± 1.29	2.04 ± 0.11
	3	36.54 ± 1.76	3.50 ± 0.09
	5	47.30 ± 1.64	4.40 ± 0.07
	7	51.34 ± 0.32	4.50 ± 0.10
	9	52.10 ± 1.04	4.56 ± 0.13

Adventitious roots were cultured in full-strength MS medium containing 30 g sucrose l^{-1} and 10 g inoculum l^{-1}

Values were obtained using five replicates, and the standard error is also shown

[a] *Control*, without plant growth regulator

Combined effect of auxin and cytokinin

In order to elucidate the optimum concentrations of kinetin and TDZ for the production of secondary metabolites, adventitious roots were treated with different concentrations of kinetin and TDZ supplemented with 5 mg l^{-1} IBA in shake flasks. The fresh weights and dry weights of *M. citrifolia* adventitious roots were significantly suppressed by different concentrations of kinetin and TDZ when supplemented with 5 mg l^{-1} IBA (Table 3). The maximum fresh weight (55.16 g l^{-1}) and dry weight (4.88 g l^{-1}) of the roots were obtained at 5 mg l^{-1} IBA (control). The fresh and dry weights of the roots decreased sharply, particularly with increasing TDZ levels. Cytokinin can inhibit root growth, especially under dark conditions. In contrast, the inhibitory effect of kinetin on root growth may be due to a reduction in the auxin content of the root (Evans 1984). The fact that the auxin–cytokinin combinations applied decreased root dry weights in this study may be due to interactions among these growth regulators impacting on

Table 2 Effects of the type and concentration of auxin on the total anthraquinone, phenolic and flavonoid contents in *Morinda citrifolia* adventitious root after 4 weeks

Treatments	Concentration (mg l^{-1})	Anthraquinone (mg g^{-1} dw)	Phenolics (mg g^{-1} dw)	Flavonoids (mg g^{-1} dw)
Control	0	50.35 ± 0.06	23.72 ± 0.35	45.00 ± 0.79
IBA	1	63.10 ± 0.09	29.46 ± 0.68	60.23 ± 0.97
	3	32.32 ± 0.35	22.66 ± 0.71	30.43 ± 0.06
	5	20.39 ± 0.18	9.20 ± 0.65	20.75 ± 0.18
	7	33.58 ± 0.05	12.03 ± 0.25	30.25 ± 0.27
	9	39.67 ± 0.33	16.65 ± 0.71	36.11 ± 0.59
NAA	1	55.60 ± 0.28	22.97 ± 0.22	49.47 ± 0.54
	3	41.27 ± 0.10	14.35 ± 0.27	36.47 ± 0.38
	5	31.16 ± 0.14	8.65 ± 0.05	28.44 ± 0.39
	7	23.38 ± 0.05	6.55 ± 0.25	21.43 ± 0.43
	9	15.24 ± 0.03	4.54 ± 0.18	14.73 ± 0.59

Adventitious roots were cultured in full-strength MS medium containing 30 g sucrose l^{-1} and 10 g inoculum l^{-1}

Values were obtained using three replicates, and the standard error is also shown

Table 3 Combined effect of auxin and cytokinin on the adventitious root growth of *Morinda citrifolia* after 4 weeks

PGR	Concentration (mg l^{-1})	Fresh weight (g l^{-1})	Dry weight (g l^{-1})	Growth ratio[a]
Control (IBA)	5.0	55.16 ± 1.27	4.88 ± 0.04	4.07
Control + kinetin	0.1	35.12 ± 2.17	2.88 ± 0.14	2.40
	0.3	26.50 ± 2.40	2.40 ± 0.20	2.00
	0.5	25.66 ± 2.24	2.28 ± 0.22	1.90
Control + TDZ	0.1	21.00 ± 0.81	2.06 ± 0.08	1.72
	0.3	13.46 ± 0.77	1.54 ± 0.05	1.28
	0.5	10.30 ± 0.57	1.34 ± 0.07	1.12

Adventitious roots were cultured in full-strength MS medium containing 30 g sucrose l^{-1} and 10 g inoculum l^{-1}

Values were obtained using five replicates, and the standard error is also shown

PGR Plant growth regulator

[a] Growth ratio = harvested dry weight (g)/inoculated dry weight (g)

Table 4 Combined effect of auxin and cytokinin on the secondary metabolite contents of adventitious roots of *Morinda citrifolia* after 4 weeks of culture

PGR	Concentration (mg l^{-1})	Anthraquinones (mg g^{-1} dw)	Phenolics (mg g^{-1} dw)	Flavonoids (mg g^{-1} dw)
Control (IBA)	5.0	13.16 ± 0.01	17.89 ± 0.75	14.27 ± 0.49
Control + kinetin	0.1	13.47 ± 0.01	20.14 ± 0.13	15.55 ± 0.24
	0.3	17.07 ± 0.01	21.83 ± 0.02	18.40 ± 0.99
	0.5	15.02 ± 0.02	20.35 ± 0.24	16.91 ± 0.76
Control + TDZ	0.1	20.57 ± 0.02	27.35 ± 0.61	23.54 ± 0.93
	0.3	27.78 ± 0.03	29.57 ± 0.76	33.05 ± 0.50
	0.5	30.90 ± 0.40	29.98 ± 0.20	36.61 ± 0.61

Adventitious roots were cultured in full-strength MS medium containing 30 g sucrose l^{-1} and 10 g inoculum l^{-1}

Values were obtained using three replicates, and the standard error is also shown

PGR plant growth regulator

root growth. However, the combinations of auxin and cytokinin resulted in significant increases in secondary metabolite content, especially when the auxin was combined with different levels of TDZ (Table 4). When increasing cytokinin levels were combined with IBA, the contents of total anthraquinone, phenolics and flavonoids also gradually increased. This increasing trend was more conspicuous in the cultures containing different levels of TDZ compared to those with kinetin. The maximum contents of total anthraquinone (30.90 mg g^{-1} DW), total phenolics (29.98 mg g^{-1} DW), and total flavonoids (36.61 mg g^{-1} DW) were achieved when 0.5 mg l^{-1} TDZ were combined with the control. Phenolic compounds are considered to be secondary metabolites that are synthesized in plants through the phenylpropanoid pathway and function as a defense mechanism that reacts to various biotic and abiotic stress conditions (Dixon and Paiva 1995). In addition to this, oxidative stress also plays important roles in the production of secondary metabolites in plants. Ali et al. (2005b, 2006) showed that H_2O_2 and O_2^- induced ginsenoside content in adventitious roots of *Panax ginseng*. TDZ, a substituted urea compound, is also known to be a synthetic growth regulator that acts by modulating endogenous plant growth regulators, either directly or as a result of stress induction such as callus induction, inducing defoliation and plantlet differentiation (Murthy et al. 1998). The accumulation of anthraquinones was increased by about 134% in TDZ-treated roots and by 29% in kinetin-treated roots compared to the control. At 0.5 mg g^{-1} TDZ, the H_2O_2 content increased about fourfold compared to the control, and at the same concentration of TDZ, the anthraquinone, phenolic and flavonoid contents also increased. A similar induction of anthraquinone content was also noted with increasing H_2O_2 levels in *M. elliptica* cell cultures (Chong et al. 2005). H_2O_2 is known to be the signal that induces antioxidant defense systems in plants in response to biotic and abiotic stresses (Neil et al. 2002a, b). This suggests that the TDZ-induced H_2O_2 accumulation observed in the present study may play a significant role in the production of anthraquinones. Phenols and flavonoids

are polyphenolic compounds that are ubiquitous in nature (e.g., quercetin, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones). Polyphenolics have aroused considerable interest recently because of their potential beneficial effects on human health: they have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities. Our results for the total phenolic content show that phenolics are higher in TDZ-treated roots than in kinetin-treated roots. Kinetin-treated roots showed about a 22% increase in phenolics and a 29% increase in flavonoids compared to the control. In contrast, TDZ-treated roots showed about a 67% increase in phenolics and a 156% increase in flavonoids compared to the control. These results suggest that TDZ should be taken into account for the production of polyphenolics in adventitious roots. Yuan et al. (2001) also suggested that oxidative stress associated taxol production in suspension cultures of *Taxus chinensis* var. mairei is induced by oligosaccharide from *Fusarium oxysprum*. DPPH is a radical-generating substance and can be considered a useful method of investigating the free radical scavenging activities of phenolic compounds (Ali et al. 2005b). TDZ resulted in a significant increase in the DPPH activity (by more than 10%) when the roots were treated with 5 mg l^{-1} IBA compared to the control (Fig. 1). An increase in DPPH activity was also noted with 0.3 mg l^{-1} kinetin in combination with 5 mg l^{-1} IBA. In our study, when kinetin and TDZ were used, the accumulation of phenolics and flavonoids was correlated to the DPPH activity. The high positive correlation observed between DPPH activity and TDZ treatment (phenols, $r = 0.998$; flavonoids, $r = 0.997$) compared to kinetin

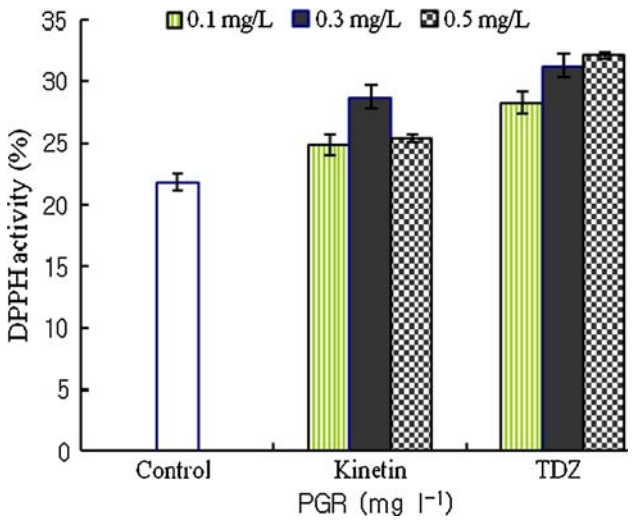

Fig. 1 Effects of different levels of kinetin and TDZ on the DPPH activity of *Morinda citrifolia* adventitious roots after 4 weeks. Control: 5 mg IBA l^{-1} was added in combination with different levels of kinetin and TDZ

treatment (phenols, $r = 0.984$; flavonoids, $r = 0.973$) suggests that TDZ plays an important role in the accumulation of phenolic compounds. It seems that the production of polyphenolics including anthraquinone observed in our study may be associated with oxidative stress.

In the present study, significant differences in antioxidant enzyme activities were observed in the roots grown in a culture medium containing different levels of kinetin and TDZ (Fig. 2). The highest CAT activities were observed at 0.3 mg l^{-1} kinetin followed by 0.5 mg l^{-1} TDZ combined with 5 mg l^{-1} IBA compared to the respective control. G-POD activity was highest at 0.1 mg l^{-1} kinetin followed by 0.5 mg l^{-1} TDZ. In contrast to CAT and G-POD, APX activity decreased significantly upon both the kinetin and the TDZ treatments compared to that of the control, and APX activity strongly decreased with increasing levels of TDZ compared to kinetin. On the other hand, no change in APX activity was noted for different levels of kinetin. Similarly, the H_2O_2 content increased with kinetin treatment compared to the control, but no variation was observed with different levels of kinetin. On the other hand, the H_2O_2 content increased significantly with TDZ treatment compared to the control, and it increased gradually with increasing concentration of TDZ. Researchers have suggested that plants activate their defense systems by altering antioxidant molecule levels and inducing antioxidative enzymes to combat oxidative stress (Noctor and Foyer 1998). Antioxidant defense enzymes such as APX, CAT and G-POD are systems that are designed to minimize the concentrations of ROS. H_2O_2 is eliminated by CAT and G-POD (Mittler 2002). CAT dismutates H_2O_2 into water, whereas G-POD decomposes H_2O_2 by oxidizing co-substrates such as phenolic compounds and/or antioxidants. On the other hand, our results showed that APX activity is inhibited with kinetin and TDZ treatment, and APX activity decreased severely with TDZ treatment compared to kinetin treatment. The marked decline in APX activity with TDZ treatment may be due to the generation of high levels of antioxidative stress, including an accumulation of higher concentrations of H_2O_2, resulting in the possible inactivation of APX activity in the treated root tissues. In contrast to this, APX activity was decreased but no variation was observed with different levels of kinetin, and a similar trend of H_2O_2 accumulation was noted with kinetin treatment. This suggests that the low inhibition of APX and low accumulation of H_2O_2 compared to TDZ treatment may be due the activation of glutathione cycle enzymes such as glutathione reductase and glutathione peroxidase enzymes, as both of these have the ability to break down H_2O_2. Inhibition of APX activity has also been noted in several plants under different treatments (Mittler 2002). Increased CAT activity is responsible for the removal of excess H_2O_2 under 0.3 mg l^{-1} kinetin and

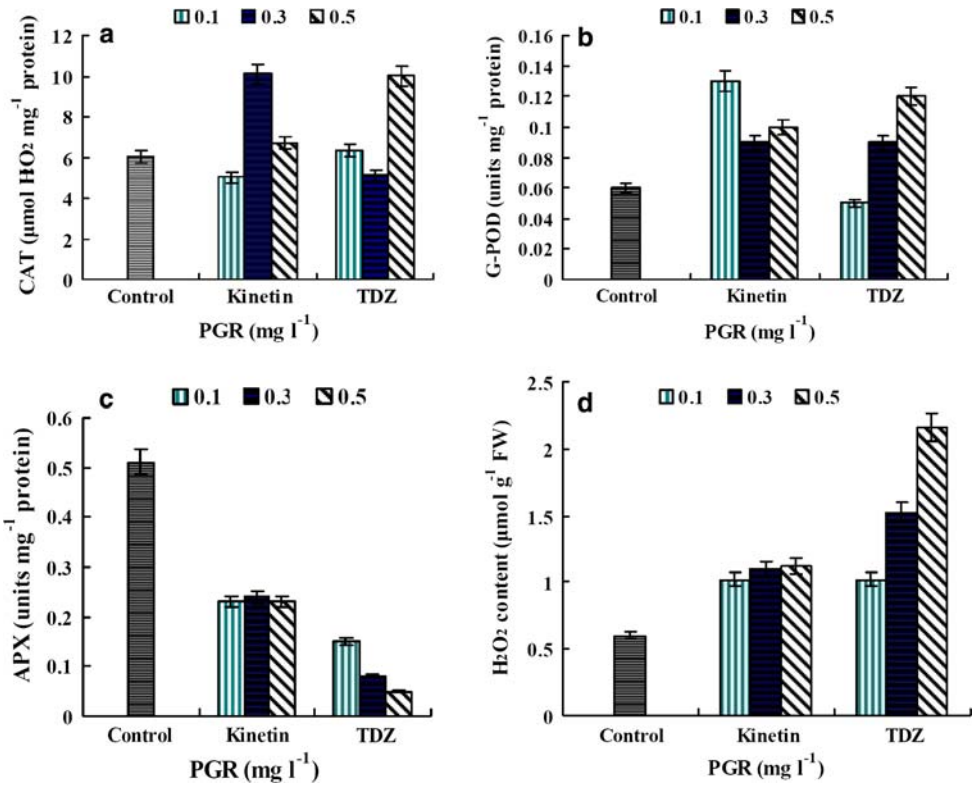

Fig. 2 Activities of the antioxidant enzymes CAT (**a**), POD (**b**) and APX (**c**) and H_2O_2 content (**d**) in adventitious roots of *Morinda citrifolia*, as affected by auxin and cytokinin (Control: 5 mg IBA l^{-1} was added in combination with different levels of kinetin and TDZ)

0.5 mg l^{-1} TDZ, while G-POD is responsible at all kinetin levels and at 0.3 and 0.5 mg l^{-1} TDZ, as indicated by the increasing CAT and G-POD activities in the present study. These results clearly indicate that 5 mg l^{-1} IBA in combination with 0.5 mg l^{-1} TDZ could be used for the production of dry root mass and secondary metabolites on a large scale using adventitious roots of *M. citrifolia*.

Conclusion

The adventitious root culture system is an efficient method of producing useful bioactive compounds. Our study suggests that 0.3 and 0.5 mg l^{-1} TDZ supplemented with 5 mg l^{-1} IBA are suitable conditions for the production of anthraquinones, including polyphenolic compounds. Our results also show that an enhancement of secondary metabolites is associated with oxidative stress, as was evidenced by the higher accumulation of H_2O_2 observed upon TDZ treatment. This study should be a useful tool in the biotechnological application of *M. citrifolia* adventitious root culture for the production of anthraquinone, phenolics and flavonoids.

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