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# Elicitor effect of chitosan and pectin on the biosynthesis of anthraquinones, phenolics and flavonoids in adventitious root suspension cultures of *Morinda citrifolia* (L.)

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**Abstract**

In this study, the effect of chitosan and pectin on the accumulation of anthraquinones (AQ), phenolics and flavonoids was investigated by adventitious root suspension cultures of *Morinda citrifolia* in shake flasks. Adventitious root cultures elicitor treated with various combinations of chitosan and pectin or chitosan alone resulted in enhanced biosynthesis of secondary metabolite but inhibited root growth. The strong inhibition of root growth might be due to the lethal effect of elicitor as evidenced by 36-79% cell death was measured. The optimum concentration of elicitor for enhancing metabolite biosynthesis was found at the concentration of 0.2 mg $ml^{-1}$ chitosan, in which 103.16, 48.57 and 75.32 mg $g^{-1}$ dry weight (DW) of AQ, phenolics and flavonoids, respectively were achieved. To elucidate the optimum contact period and reduction of inhibitory effect of chitosan on root growth, a two-stage culture system was adopted. The optimum contact period of root to elicitor was observed when adventitious culture was elicitor treated with 0.2 mg $ml^{-1}$ chitosan on day 28 and harvest after 2 days of elicitation. Although, root growth slightly decreased by this treatment, the yield of AQ, phenolics and flavonoids was enhanced by 45%, 8% and 12%, respectively compared to without elicitor treated culture.

**Keywords:** Adventitious root, anthraquinones, chitosan, flavonoids, pectin, phenolics, two-stage culture.
**Abbreviations:** AQ- anthraquinones; IBA- indole butyric acid; MeOH- methanol; MS- murashige and skoog; SDS- sodium dodecyl sulfate.

## Introduction

Plant cell and root cultures can be established routinely under sterile conditions from explants, such as plant leaves, roots or stems. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement of secondary metabolite production. However, most often trails with plant cell or root cultures fail to produce the target product. In such cases, strategies to enhance production of desired metabolites must be considered (Dornenburg and Knorr, 1995; Kim et al., 2005a; Jeong et al., 2009). With this context, several techniques have been adopted to improve production of plant-derived secondary metabolites such as elicitation, precursor feeding, two-phase and two-stage culture system, genetic transformation, metabolic engineering etc. (Hahn et al., 2003; Chong et al., 2005; Zhou and Zhong, 2011). Of the various efforts, elicitation is seen as an effective strategy to enhance production of commercially important bioactive compounds such as AQ (Komaraiah et al., 2005), ginsenosides (Paek et al., 2009), paclitaxel and other taxanes (Zhong, 2002), saikosaponin (Chen et al., 2007) and scopolamine (Biondi et al., 2000). Elicitors are biotic and abiotic compound triggering the formation of secondary metabolites. The use of biotic or abiotic elicitors to stimulate product formation has become an important progress strategy and has been very useful in reducing the process time required to attain high product concentrations and increased volumetric productivity (Cai et al., 2011). Natural elicitors include polysaccharides such as pectin and chitosan, which are being frequently used in a number of plant cell cultures for efficient induction of pharmaceutically important secondary metabolites. Chitosan is an exogenous biotic elicitor that is derived from the fungal cell wall (Montesano et al., 2003), has been studied for their effects on phenylpropanoid metabolic enzymes (Chakraborty et al., 2009) and secondary metabolite production (Orlita et al., 2008; Wiktorowska et al., 2010). Pectin was also found to enhance induction of oleanolic acid biosynthesis in cell suspension cultures of *Calendula officinalis* (Wiktorowska et al., 2010). Chitosan and pectin involved in interactions between plants and microorganisms gave the best results by activating secondary metabolism in *M. citrifolia* cell suspension cultures (Dornenburg and Knorr, 1995). Moreover, it has been demonstrated that the addition of pectin to chitosan-treated cell cultures had positive effects on cell growth through providing protective mechanism against cell damage and amaranthin accumulation in *Chinopodium rubrum* suspension cells (Dornenburg and Knorr, 1995). Therefore, considering the beneficial effect of pectin in protecting cell damage, various concentrations of pectin were added in adventitious root suspension cultures of *M. citrifolia* in combination with chitosan. Results revealed that pectin did

not show any protective mechanism against chitosan induced cell damage. *M. citrifolia* (L.) is recognized as a high value medicinal plant due to a broad range of therapeutic effects, including anticancer, antiviral, antibacterial, anti tumor, treatment of cold and flue, analgesic, hypotensive, anti-inflammatory and immune enhancing effects (Wang et al., 2002). The main ingredients of *M. citrifolia* are polyphenolics, organic acids and alkaloids, and of the polyphenolics the mostly reported ones are AQ (Wang and Su, 2001; Wang et al., 2002). The cell suspension cultures of *M. citrifolia* and its other species for the production of AQ have been reported by several authors (Zenk et al., 1975; Abdullah et al., 2000; Chong et al., 2004, 2005; Komariah et al., 2005; Ahmed et al., 2008; Deshmukh et al., 2011). However, low content of AQ and high water content in cells are observed as main obstacle for large scale production. As one of alternative approach, we have established adventitious root suspension culture of *M. citrifolia*. The induction of adventitious roots, optimization of plant growth regulators, Murashige and Skoog (MS) medium dilutions and sucrose concentrations for AQ, phenolics and flavonoids production has previously been reported (Baque et al., 2010a, 2010b, 2010c, 2011b). Considering potential benefit of chitosan and pectin for efficient induction of various plant secondary metabolites including AQ, in this study, we have elucidated the involvement of chitosan and pectin on adventitious root growth and biosynthesis of AQ, phenolics and flavonoids. Besides, the protective effect of pectin against cell death was also studied. After selecting suitable elicitor and its concentration, optimum elicitor contact period was also investigated by applying two-stage culture system. To the best of our knowledge, this is the first time that the elicitor effect of chitosan and pectin on the accumulation of biomass and bioactive compounds in adventitious root suspension cultures of *M. citrifolia* is reported.

**Results and discussion**

***Combined effect of chitosan and pectin on adventitious root growth and bioactive compounds production***

Several combinations of chitosan and pectin at various concentrations were performed to determine their effect on the accumulation of root biomass and biosynthesis of AQ, phenolics and flavonoids. Adventitious root suspension cultures exposed to various concentrations and combinations of chitosan and pectin during inoculation resulted in a significant reduction in root growth (Table 1). With increasing chitosan and pectin ratio root fresh weight (FW), dry weight (DW) and growth ratio (GR) significantly decreased. A more than 5.57-fold FW and 3.73-fold DW reduction were recorded when adventitious root cultures were elicitor treated with 0.8/0.8 mg $ml^{-1}$ chitosan and pectin compared to the relative control. The strong inhibition of root growth by elicitor treatment with chitosan and pectin was found to be associated with strong inhibition of cell viability (Fig. 1). Over 79% induction of root death was noted when adventitious roots were exposed to 0.8/0.8 mg $ml^{-1}$ chitosan and pectin. The accumulation of AQ, phenolics and flavonoids showed a dose- dependent response to chitosan and pectin treatments (Table 2). Adventitious root cultures exposed to 0.2 mg $ml^{-1}$ chitosan induced maximum accumulation of AQ (103.16 mg $g^{-1}$ DW) and flavonoids (75.32 mg $g^{-1}$ DW). While the highest accumulation of phenolics (52.45 mg $g^{-1}$ DW) was detected when adventitious root culture exposed to 0.2 mg $ml^{-1}$ chitosan in combination with 0.2 mg $ml^{-1}$ pectin. At the concentration above 0.2 mg $ml^{-1}$ chitosan, the accumulation of AQ, the major bioactive compound decreased. This result suggests that 0.2 mg $ml^{-1}$ chitosan is an optimal concentration for uplifting AQ biosynthesis. Although, the addition of pectin with chitosan at various concentrations induced biosynthesis of those metabolites compared to control, however, strong inhibition of root growth was occurred. This means that addition of pectin in combination with chitosan did not show any protective effect against elicitor-induced cell damage. Chitosan and pectin which are a group of biotic elicitors have frequently been used in a number of plant cell cultures for efficient induction of plant secondary metabolites, including AQ. It has been reported that cultures elicitor treated with chitosan efficiently enhanced biosynthesis of isoflavonoid in *Pueraria candollei* var. and oleanolic acid in *Calendula officinalis* (Wiktorowska et al., 2010), rosmarinic acid in *Ocimum basilicum* (Kim et al., 2005b), indirubin in *Polygonum tinctorium*, phytoalexins in *Nicotiana tobaccum*, genistein in *Lupinus albus* and anthracene in *Rheum palmatum* (reviewed by Aijaz et al., 2011), anthocyanin and phenolic acid in *Vitis vinifera* (Cai et al., 2011), AQ in *Rubia akane* Nakai (Jin et al., 1999) and in *Morinda citrifolia* suspension cells (Dornenburg and Knorr, 1995). Similarly, pectin was found responsible for enhancing oleanolic acid accumulation in *Calendula officinalis* cell suspension cultures (Wiktorowska et al., 2010). It has been demonstrated that the addition of pectin to chitosan-treated cell cultures had positive effects on cell growth and amaranthin accumulation in *Chenopodium rubrum* cells (Dornenburg and Knorr, 1995). In addition, the protective effect of pectin was also observed in *M. citrifolia* cell suspension cultures when combinedly treated with chitosan (Dornenburg and Knorr, 1995). Conversely, viability studies with *C. rubrum* suspension cultures, chitosan solutions showed lethal effects at concentrations >400 µg chitosan $ml^{-1}$ medium. Increased chitosan concentration caused an increase in pigment release, which was correlated with cell death (Dornenburg and Knorr, 1995). It has been reported that if the changes in gene expression include decreased total protein synthesis, induction of hypoxia-associated proteins, and glucose regulated proteins are inadequate to prevent ATP depletion. Membrane ion pumps fail and membrane integrity is lost. Increased intercellular $Ca^{2+}$ occurs and varieties of degradation processes are initiated, leading to cytoplasomic swelling and eventually cell death (Cobb et al., 1996). In this study, we observed lethal effects of chitosan on root growth at concentrations > 0.2 mg $ml^{-1}$, as evidenced by 60-79% cell death was detected (Fig. 1). Although, combination of chitosan and pectin at various concentrations enhanced induction of AQ, phenolics and flavonoids biosynthesis compared to the relative control, however strong inhibition of root growth was measured. This suggests that exogenous addition of pectin in combination with chitosan did not show any protective effect on cell viability. Myriad researches in plant biotechnology have suggested that adventitious root cultures exposed to elicitor treatment resulted in a strong reduction in root growth but enhanced biosynthesis of bioactive compounds (Hahn et al., 2003; Kim et al., 2005a; Ali et al., 2006; Paek et al., 2009). The adaptation of two-stage culture system: adventitious roots of ginseng cultured with elicitor free medium for the first 40 days and subsequently elicitor treated with methyl jasmonate for last 10 days, effectively enhanced biosynthesis of ginsenosides without affecting root growth (Kim et al., 2005a). Therefore, considering the potential benefit of two-stage culture system, 0.2 mg $ml^{-1}$ chitosan (the best concentration for inducing secondary metabolism) was selected for further investigation.

**Table 1.** Combined effect of chitosan and pectin on adventitious root growth of *Morinda citrifolia* after 4 weeks of culture.

| Chitosan/Pection (mg $ml^{-1}$) | Fresh weight (g $l^{-1}$) | Dry weight (g $l^{-1}$) | Growth ratio |
|---|---|---|---|
| Control | 47.68±0.118a | 4.48±0.047a | 4.71 |
| 0.2/0 | 31.22±1.99b | 3.03±0.154b | 3.18 |
| 0.2/0.1 | 11.55±0.064d | 1.30±0.040de | 1.37 |
| 0.2/0.2 | 11.58±0.228d | 1.30±0.070de | 1.37 |
| 0.4/0.2 | 9.98±0.649e | 1.25±0.028de | 1.32 |
| 0.4/0.4 | 11.80±0.681c | 1.48±0.085c | 1.55 |
| 0.8/0.8 | 8.55±0.086f | 1.20±0.040e | 1.26 |

Mean separation within columns by Duncan's multiple range tests at 5% level. The various combinations of chitosan and pectin were added in the culture during inoculation. Control = without chitosan and pectin.

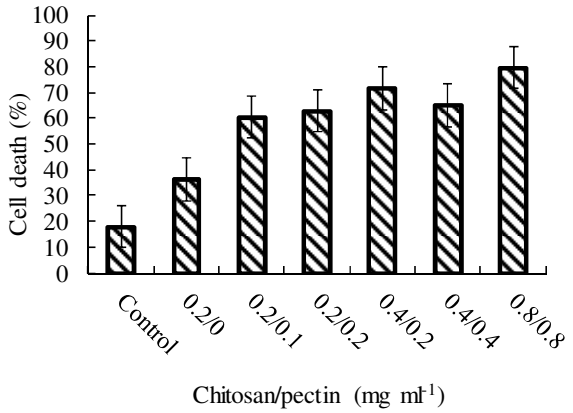


**Fig 1.** Combined effect of chitosan and pectin on cell death (%) of Morinda citrifolia adventitious roots after 4 weeks of culture. Various concentrations of chitosan and pectin were added in the culture during inoculation. Bars represent means ± SE (n = 4).

**Table 2.** Combined effect of chitosan and pectin on the accumulation of secondary metabolites in adventitious roots of *Morinda citrifolia* after 4 weeks of culture

| Chitosan/Pectin (mg $ml^{-1}$) | AQ (mg $g^{-1}$ Dw) | Phenolics (mg $g^{-1}$ Dw) | Flavonoids(mg $g^{-1}$ Dw) |
|---|---|---|---|
| Control | 49.30±0.420g | 24.19±0.023g | 41.44±0.170f |
| 0.2/0 | 103.16±0.242a | 48.57±0.187c | 75.32±0.124a |
| 0.2/0.1 | 98.90±0.182b | 50.45±0.564b | 65.89±0.047b |
| 0.2/0.2 | 93.33±0.113c | 52.45±0.049a | 61.22±0.084c |
| 0.4/0.2 | 64.293±0.197e | 42.01±0.024e | 45.44±0.508e |
| 0.4/0.4 | 67.40±0.219d | 42.69±0.187d | 48.04±0.299d |
| 0.8/0.8 | 50.94±0.151f | 33.88±0.199f | 35.89±0.057g |

Mean separation within columns by Duncan's multiple range tests at 5% level. The various combinations of chitosan and pectin were added in the culture during inoculation. Control = without chitosan and pectin.

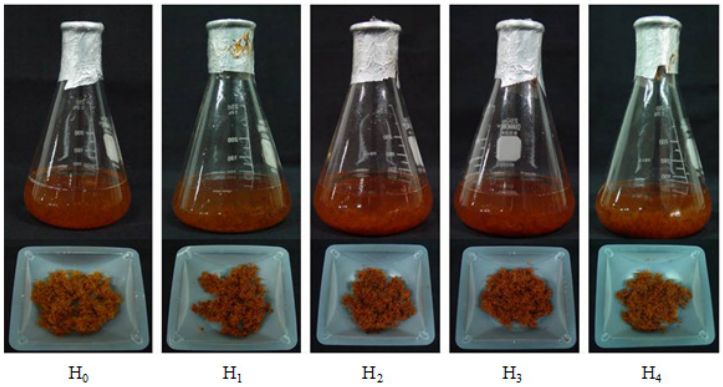


**Fig 2.** Effect of chitosan on adventitious root growth of Morinda citrifolia harvested after different days of elicitation. (0.2 mg ml-1 chitosan was added in the culture on day 28). H0 (without chitosan): Harvested on day 28; H1: Harvested on 2 days after elicitation; H2: Harvested on 4 days after elicitation; H3: Harvested on 6 days after elicitation; H4: Harvested on 8 days after elicitation. (Adventitious root growth of Morinda citrifolia decreased with increasing exposure time to 0.2 mg ml-1 of chitosan).

Results reveal that as application of two-stage culture system is an effective strategy for uplifting productivity of bioactive compounds in adventitious root suspension cultures of *M. citrifolia*.

***Effect of chitosan as two-stage culture system on the accumulation of root biomass and bioactive compounds***

To elucidate the optimum exposure time of roots to elicitor for enhancing metabolites biosynthesis and reducing inhibitory effects of elicitor to root growth, adventitious root cultures were exposed to 0.2 mg $ml^{-1}$ chitosan on day 28 and harvest after different days (0-8) of elicitation. Results depicted in Fig. 2 and Table 3 show that with increasing exposure time of roots to 0.2 mg $ml^{-1}$ chitosan, root FW, DW and GR profusely decreased. Contrary, the accumulation of AQ, phenolics and flavonoids increased on day 2, after that gradually decreased up to day 6 followed by increased up to day 8 (Table 4). Although, cultures exposed to 0.2 mg $ml^{-1}$ chitosan slightly inhibited root growth (Table 3), the highest yield of AQ (332.24 mg $l^{-1}$ DW), phenolics (121.27 mg $l^{-1}$ DW) and flavonoids (212.24 mg $l^{-1}$ DW) observed after 2 days of elicitation (Table 4). This might be due to 1.65-, 1.23- and 1.28-fold increases of AQ, phenolics and flavonoids accumulation, respectively after 2 days of elicitation compared to the relative control. Interestingly, the biosynthesis of AQ was more activated by chitosan treatment than phenolics and flavonoids. Indicating that chitosan is an effective elicitor for efficient induction of AQ biosynthetic pathway than phenylpropanoid pathway that leads to enhance biosynthesis of AQ. Elicitor contact period is an important factor, because a critical contact period could be favorable for the mass production of secondary metabolites. In suspension cultures of madder cells of *Rubia akane* Nakai, AQ production was increased in proportion to the contact period of 25 mg $l^{-1}$ chitosan up to day 3, and no further increase was observed after 3 days of elicitation (Jin et al., 1999). The exogenous addition of 200 mg $l^{-1}$ chitosan was found beneficial for enhancing indirubin production (72%) in suspension cultures of *Polygonum tinctorium* when cells were exposed to chitosan for 5 days (Kim et al., 1997). The enhanced induction of AQ in cell suspension cultures of *M. elliptica* was also achieved by elicitor treatment with 100 µM jasmonic acid for 3 days. Cultures exposed to 100 µM jasmonic for 6 days inhibited biosynthesis of AQ, instead induced oxidative stress (Chong et al., 2005). In this study, the optimum contact period of adventitious roots to 0.2 mg $ml^{-1}$ chitosan for enhancing biosynthesis of AQ, phenolics and flavonoids was noticed when roots were exposed to elicitor for 2 days. Adventitious root cultures exposed to chitosan for 6 days significantly inhibited accumulation of AQ and flavonoids (Table 4). By contrast, the accumulation of phenolics increased in proportion to the contact period of chitosan up to day 8. Adventitious root cultures exposed to chitosan for 8 days enhanced biosynthesis of AQ and flavonoids but still lower than that of control treatment (Table 4), as well as strong inhibition of root growth was detected (Table 3). The present investigation is partially inconsistent with our previous study, in which adventitious root cultures elicitor treated with 150 µM methyl jasmonate (MeJa) on day 28 and harvest after 4 days of elicitation enhanced biosynthesis of AQ, phenolics and flavonoids (Baque, 2011). Cultures exposed to MeJa over 4 days of elicitation resulted in induction of oxidative stress and peroxidation of membrane lipids that leads to reduction of root growth and accumulation of bioactive compounds. In root suspension cultures of *Panax ginseng*, the strong inhibition of root growth but enhanced accumulation of phenolics and flavonoids was observed by MeJa and salicylic acid-induced oxidative stress when cultures were exposed to elicitor up to day 9 (Ali et al., 2007). Lending from this information it is tempting to speculate that adventitious root cultures exposed to 0.2 mg $ml^{-1}$ chitosan over 2 days might be induced lethal oxidative stress that strongly inhibited root growth and biosynthesis of bioactive compounds. These results indicate that adventitious root cultures of *M. citrifolia* exposed to 0.2 mg $ml^{-1}$ chitosan over 2 days is not beneficial for both biomass and bioactive compound production.

**Materials and methods**

***Plant material, induction and proliferation of adventitious roots***

Adventitious roots were induced from leaf explants of *in vitro* grown plantlets of *M. citrifolia*. The plantlets were grown from mature seeds after sterilized with a 4% sodium hypochlorite solution for 20 min followed by soaking in a 2% sodium hypochlorite for 10 min in a laminar hood. After that seeds were washed in sterile distilled water until the sterilizer was completely removed and clipped using a sterilized clipper. Seeds were inoculated in test tubes containing 10 ml of MS medium without growth regulator. The leaf-originated adventitious roots (Baque et al., 2010a), were proliferated in liquid MS media supplemented with 5 mg $l^{-1}$ IBA, 3% sucrose and 10 g $l^{-1}$ inoculum size. Cultures were agitated in 100 rpm on a gyratory shaker under darkness at 23±2°C for 4 weeks. Adventitious roots were subcultured using same media after every four weeks.

***Combined effect of chitosan and pectin***

To determine optimum concentration and combination of chitosan and pectin on root growth and secondary metabolites accumulation, various combinations of chitosan and pectin (0, 0.2/0, 0.2/0.1, 0.2/0.2, 0.4/0.2, 0.4/0.4 and 0.8/0.8 mg $ml^{-1}$) were added to the medium during inoculation. Chitosan solution was prepared in aqueous acidic solution according to Varum *et al*. (1994) and pectin solution in double-distilled water. A 15 g $l^{-1}$ fresh adventitious root was inoculated in 250 ml flask containing 100 ml quarter- strength MS medium supplemented with 5 mg $l^{-1}$ IBA and 1 % sucrose. Cultures were agitated in 100 rpm on a gyratory shaker under darkness at 23±2°C for 4 weeks (Baque et al., 2010b, 2010c, 2011b).

***Effect of chitosan as two-stage culture system***

To explore the optimum contact period of chitosan to roots for enhancing accumulation of secondary metabolites, adventitious roots were cultured in 250 ml shake flask (using the same medium mentioned above). The adventitious roots were allowed to grow for 4 weeks without elicitor. After 4 weeks of culture, 0.2 mg $ml^{-1}$ of chitosan (regarded as best concentration from previous experiment) was added to the medium and harvested after 0, 2, 4, 6 and 8 days of elicitation. All the cultures were agitated in 100 rpm on a gyratory shaker and kept under darkness at 23±2°C.

***Growth measurement***

The harvested roots from each treatment were washed several times with distilled water, soaked with tissue paper to remove the surface water and fresh weight (FW) was measured. For dry weight (DW), adventitious roots were dried at 60° C for

**Table 3.** Effect of chitosan on adventitious root growth of *Morinda citrifolia* (0.2 mg/ml chitosan was added in the culture on day 28).

| Harvesting time | Fresh weight ($g\ l^{-1}$) | Dry weight ($g\ l^{-1}$) | Growth ratio |
|---|---|---|---|
| $H_0$ | 46.00±0.912a | 4.45±0.064a | 4.68 |
| $H_1$ | 39.55±0.476b | 3.90±0.108b | 4.11 |
| $H_2$ | 32.73±0.838c | 3.13±0.075c | 3.29 |
| $H_3$ | 30.05±1.951d | 2.18±0.103d | 2.29 |
| $H_4$ | 28.05±0.777e | 1.85±0.028e | 1.95 |

Mean separation within columns by Duncan's multiple range tests at 5% level. $H_0$ (without chitosan): Harvested on day 28; $H_1$: Harvested on 2 days after elicitation; $H_2$: Harvested on 4 days after elicitation; $H_3$: Harvested on 6 days after elicitation; $H_4$: Harvested on 8 days after elicitation.

**Table 4.** Effect of chitosan on the production of secondary metabolites in adventitious root of *Morinda citrifolia* (0.2 mg/ml chitosan was added in the culture on day 28).

| Harvesting time | AQ | | Phenolics | | Flavonoids | |
|---|---|---|---|---|---|---|
| | Content ($mg\ g^{-1}$ dw) | Yield ($mg\ l^{-1}$ dw) | Content ($mg\ g^{-1}$ dw) | Yield ($mg\ l^{-1}$ dw) | Content ($mg\ g^{-1}$ dw) | Yield ($mg\ l^{-1}$ dw) |
| $H_0$ | 51.37±0.349 c | 228.61±0.553b | 25.23±0.074e | 112.29±0.332b | 42.42±0.139b | 188.76±0.619b |
| $H_1$ | 85.19±0.038a | 332.24±0.148a | 31.10±0.462a | 121.27±0.804a | 54.42±0.314a | 212.24±0.224a |
| $H_2$ | 58.97±0.857b | 184.57±0.628c | 26.91±0.200d | 84.23±0.626c | 33.29±0.688c | 104.21±0.153c |
| $H_3$ | 44.81±0.597e | 97.68±0.301d | 27.22±0.062c | 59.34±0.136d | 28.29±0.462e | 61.68±1.007d |
| $H_4$ | 49.49±0.023d | 91.56±0.042e | 29.61±0.225b | 54.78±0.416e | 30.79±0.638d | 56.96±1.180e |

Mean separation within columns by Duncan's multiple range tests at 5% level. $H_0$ (without chitosan): Harvested on day 28; $H_1$: Harvested on 2 days after elicitation; $H_2$: Harvested on 4 days after elicitation; $H_3$: Harvested on 6 days after elicitation; $H_4$: Harvested on 8 days after elicitation. Yield = Dry weight ($g\ l^{-1}$) * content ($mg\ g^{-1}$ DW).

72 h or more until a constant weight. The growth ratio (GR) was measured as:
GR = {harvested DW (g) – inoculated DW (g)} / inoculated DW (g).

***Extraction and determination of AQ***

Roots were collected after harvest from each treatment and washed with distilled water and dried at 60 $^{o}$ C for 72 h or more until a constant weight. The dried root sample (0.2 g) was digested in 40 ml of 80% ethanol using Cod Oil Bath digestion system (LS-2050-S10, Tech, Korea) for 2 h at 80$^{o}$C and filtered through filter paper (Advantic, 110 mm, Japan). The procedure was repeated until final extractant was colorless and adjusted to 50 ml by adding rest amount of 80% ethanol. The ethanol fractions were pooled and filtered by using 0.45 µm whatman micro filter. AQ was determined by a spectrophotometer (UV-1650PC, Shimadzu, Japan) at 434 nm using alizarin (Sigma, Germany) as standard (Zenk et al., 1975).

***Determination of phenolics and flavonoids***

The contents of total phenolics and flavonoids were analyzed spectrophotometrically from the same aforementioned root ethanolic extract according to the previously described method by Wu et al. (2006). For phenolics measurement, the ethanolic extracts (0.1 ml) were mixed with 2.5 ml deionized water, followed by the addition of 0.1 ml (2 N) Folin-Ciocaltue reagent. After the tubes were shaken thoroughly for 5 min, a 0.5 ml solution of $Na_2CO_3$ (20% w/v) was added and the mixture was allowed to stand for 30 min in the dark at room temperature. The color was developed and the absorbance was measured at 760 nm in a UV-visible spectrophotometer. The measurements were compared to a standard curve for gallic acid and were expressed as the mg of gallic acid equivalent per gram of root DW.
For flavonoids, 0.25 ml of ethanolic root extract or a (+/-) catechin standard solution was mixed with 1.25 ml of distilled water, followed by the addition of 75 µl of a 5% (w/v) sodium nitrite solution. After 6 min, 0.15 ml of 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for further 5 min before 0.5 ml of 1 M sodium hydroxide was added. The absorbance was measured immediately at 510 nm in a spectrophotometer and results were expressed as mg of catechin equivalents per gram of root DW.

***Measurement of cell death***

Cell death, indicates as loss of plasma membrane integrity, was measured spectrophotometrically as Evans blue uptake according to the method described by Baker and Mock (1994) with slight modification. The harvested roots were washed with double-distilled water. After that, 0.5g fresh roots were incubated in Evans blue solution (0.025% [w/v] Evans blue in water) for 15 min and soaked in double-distilled water for 5 min followed by centrifuging at 11000 rpm to wash the roots in order to remove excess and untrapped Evans blue. This procedure was repeated three times. The trapped Evans blue was released from the roots by homogenizing with a 1.5 ml measuring solution (50% [v/v] MeOH and 1% SDS). The homogenate was incubated for 15 min in a water bath at 50$^{o}$C and centrifuged at 14,000 rpm at 4$^{o}$C for 15 min. The optical density of the supernatant was determined at 600 nm and expressed on the basis of 100% dead cell. Fresh root samples were boiled in water at 100$^{o}$C for 30 min to make them 100% dead and the optical density of the dead roots were measured by following the same procedure.

**Statistical analysis**

The experiments were conducted in a completely randomized design with four replicates. Data were subjected to an analysis of variance (ANOVA). To determine the significant difference, Duncan's multiple range test (DMRT) was performed using SAS software (Version 6.12; SAS Institute, USA).

**Conclusion**

These results obtained from this study reveal that chitosan is a potential elicitor and application of two-stage culture

system: adventitious root cultures of *M. citrifolia* exposed to 0.2 mg $ml^{-1}$ chitosan on day 28 and harvest after 2 days of elicitation is an effective strategy for uplifting productivity of AQ, phenolics and flavonoids. However, further studies regarding combinations of chitosan and pectin at low concentrations and investigation of closer exposure time of root to elicitor, as well as elicitor-induced oxidative defense mechanism and pathway related enzymatic activities would be more interesting to explore the insight of this present work.

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