

## Pharmaceutical Communication

# Establishment of *In vitro* Adventitious Root Cultures, Analysis of Phenolics and Curculigoside Contents in *Curculigo orchioides*

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### ABSTRACT

Traditionally, *Curculigo orchioides* was used for aphrodisiac and many other effects. Curculigoside is the principal bioactive component of the medicinal plant *Curculigo orchioides*, to which various diverse pharmacological properties are attributed. Adventitious root culture of leaf explants of *Curculigo orchioides* was studied using different strength MS medium supplemented by different concentrations of sucrose and pH different values for growth and secondary compound (phenolic and curculigoside) production with 3.0 mg/L NAA in liquid culture. Adventitious roots grown in modified ¾ strength of MS medium showed the highest root growth (2.499 g/g FW), as well as the highest amount of curculigoside (76.521 µg/Treatment) as compared with roots grown in different treatments. HPLC analysis displayed the presence of curculigoside from the *in vitro* adventitious roots of *Curculigo orchioides*. The growth characteristics (biomass) together with phenolic and curculigoside content from the established *in vitro* adventitious roots of *Curculigo orchioides* was successfully enhanced by the manipulation of culture conditions involving ¾ strength of MS medium and the addition of 4% (w/v) of sucrose. Hence, it is important to study other strategies in order to enhance the maximum production of secondary metabolites in *in vitro* systems.

**KEY WORDS:** CURCULIGO ORCHIOIDES, ADVENTITIOUS ROOTS, CURCULIGOSIDE, HPLC, MEDIUM STRENGTH.

### INTRODUCTION

*Curculigo orchioides* Gaertn. (Amaryllidaceae), which grows in subtropical regions of Asia, has been used as a traditional herbal medicine in China, India and Vietnam. It is believed to be a tonic for the treatment of declined physical strength. Curculigoside, one of the main bioactive phenolic compounds in the rhizome of *Curculigo orchioides* Gaertn., has been shown to have significant antioxidant properties by scavenging superoxide radicals in the normal systems, and anti-apoptotic activities in H<sub>2</sub>O<sub>2</sub>-treated vascular endothelial cells (Tang et al. 2004; Wu et al., 2005; Wang et al., 2010).

Curculigoside reduced the oxidative damage and induced proliferation and differentiation of osteoblasts

under oxidative stress status, as well as inhibited bone resorption via its anti-oxidative character in ovariectomized rats (Wang et al., 2012; Jiao et al., 2009; Liu et al., 2012). Curculigoside increased the proliferation and alkaline phosphatase (ALP) activity of osteoblasts, inhibited osteoclast bone resorption, osteoclast formation and tartrate-resistant acid phosphatase activity (Jiao et al., 2009). Curculigoside protected osteoblasts against oxidative damage and promoted osteoblastic differentiation via inhibiting extracellular signal-regulated kinase (ERK) and ERK dependent nuclear factor-κB (NF-κB) signaling pathways and stimulating p38 signaling pathway under oxidative stress conditions (Wang et al., 2012). In addition, Curculigoside can also improve the learning and memorizing ability of aged rats by decreasing cerebral acetylcholinesterase activity and inhibiting the expression of β-site APP cleaving enzyme 1 in the hippocampus (Wu et al., 2012). Curculigoside

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effects on A $\beta$  deposition induced memory deficit, bone loss and the potential proximate mechanisms (Zhao et al., 2015).

The quality and quantity of the secondary metabolite collected from wild and fieldgrown plants are often fluctuating and heterogeneous depending upon the environmental conditions. Infestation, diseases and the application of pesticides additionally decrease the quality of the plant materials (Gerth et al., 2007). One is hence listed in this category. Therefore, there is a need to develop an alternative strategy to full-fill the ever increasing demand. Few of the known strategies are *in-vivo* cultivation, micropropagation, direct rhizogenesis and root organ culture. By these techniques, secondary metabolites are isolated and produced in large quantities, as well as of good quality and are stable and produced rapidly in an adapted culture medium. Therefore, production of secondary metabolites under *in vitro* conditions has become an active field of research (Sato et al., 2001).

Adventitious roots have been successfully induced in many plant species and cultured for the production of high value secondary metabolites of pharmaceutical, nutraceutical and industrial importance (Mehrotra et al., 2007; Murthy et al., 2008). In this study, we describe the effects of the different strength of media, concentrations of sucrose and pH different on growth, phenolics and curculigoside production in adventitious root culture *Curculigo orchiooides* Gaertn.

## MATERIAL AND METHODS

**Plant material and adventitious root induction:** Wild plants were collected in the An Giang province in Vietnam and were identified by Dr. Son of Botanical Museum (Institute of Tropical Biology, Vietnam Academy of Sciences and Technology). Young leaves were used as explants for adventitious root induction in winter. Leaf explants were washed with 70% ethanol for 30 s. And then they were transferred into the bottles with 100 mL of 50% (v/v) sodium hypochlorite solution and 5–6 drops of tween 80.

The bottles were shaken for 20 min. Leaf explants were cut into 0.5 cm×0.5 cm sections after being washed with sterilized distilled water for five times. They were inoculated on MS (Murashige and Skoog, 1962) medium containing 3.0 mg/L NAA (1-naphthaleneacetic acid), 3% (w/v) sucrose and 0.6% (w/v) agar. All the initial pHs of the medium were adjusted to 5.80 with 0.1 M of NaOH or 0.1 M of HCl before autoclaving. The cultures were maintained in a growth chamber at different temperatures 25 °C in the dark. The initiation ratios and lengths of the adventitious roots were recorded after incubation for eight weeks.

**Effects of Culture Conditions on Root Biomass:** The well established *in vitro* adventitious roots that were cultured onto MS medium supplemented with 3.0 mg/L

NAA was used as the initial material. Liquid cultures were established by inoculating 1.0 g (fresh weight, FW) adventitious roots into 100 mL Erlenmeyer flasks containing 75 mL of different strength liquid MS medium with NAA (3.0 mg/L). The flask was kept under continuous agitation at 80 rpm on an orbital shaker at 25 ± 1°C under dark condition.

The adventitious roots were transferred onto MS medium with different MS strengths (1/4, 1/2, 3/4, and 1), sucrose concentrations (0%, 2%, 3%, 4%, 5%, 6% and 7%) and the initial pHs of the medium was adjusted (to 5.5; 6.0; 6.5; 7.0; 7.5) with 1.0 M sodium hydroxide (NaOH) and/or 1.0 M hydrochloric acid (HCl) prior to autoclaving. Growth of the adventitious roots was quantified on the basis of initial weight and final weight of fresh weight, dry mass, concentration of phenolic (mg/g DW), and concentration of curculigoside (µg/g DW) after four weeks.

**Statistical analysis:** All *in vitro* experiments were conducted in 3 replications. The data were analyzed by analysis of variance (ANOVA) to detect significant differences between means using MS Excel. Means differing significantly were compared using the LSD at the 1% probability level by SAS program (ver. 6.12). Quantification of Phenolic and Curculigoside Content from Adventitious Root Cultures

**Plant Extraction:** The adventitious roots were cleaned, sliced, oven dried at 38 °C, ground into powder and extracted with the method 50% of the ultrasonic vibration. The mass of the methanol extract was recorded and re-dissolved in methanol HPLC grade at a ratio of 1 mg of extract to 100 µl methanol HPLC grade. This methanolic solution of the extract was filtered through 0.45 µm PTFE filter (Sartorius 13 CR) prior to quantification of phenolic and curculigoside content.

**Quantification of Phenolic Content:** The total phenolic content (TPC) in *Curculigo orchiooides* adventitious roots crude extracts was determined by using the Folin–Ciocalteu method (Chang et al., 2002). Standard solutions of gallic acid of concentration 1.56–100 µg/ml were prepared in water. 50 µl of extract (1 mg/ml) or standard solution were added to 50 µl of distilled water. 50 µl of 10% Folin–Ciocalteu's (F–C) phenol reagent and 50 µl of 1 M sodium carbonate solution were added to the mixture in a 96-well plate. Distilled water was used as blank. Reactions were incubated for 60 min at room temperature and protected from light. The absorbance was measured at 750 nm with a Microplate Reader (Biotek, USA.). Total phenolic contents were expressed as µg Gallic Acid Equivalents (GAE) per mg of dry plant material.

**HPLC Analysis:** The curculigoside was extracted from the crude medicine with the method 50% of the ultrasonic vibration. Using Sep-Pak C18 cartridges to purify the solution, the curculigoside was detected by HPLC. C18 (250 mm x 4,6 mm; 5 µm) chromatographic column was used, mobile phase of water–Acetonitril (78:22) and detect

wavelength was set at UV 284 nm (Lu et al., 2002). The curculigoside was detected and quantified by matching their retention times and spectral characteristics with known standards that had been identified previously.

## RESULTS AND DISCUSSION

Adventitious root formation has a complex molecular process involving numerous endogenous and exogenous physiological factors (Sorin et al. 2005). According to Praveen et al. 2009, the process of induction and differentiation in the physiological stages of rooting can be triggered by changes in endogenous auxin concentrations and external addition of specific auxins (Praveen et al. 2009). *Curculigo orchoides* leaf samples were grown on MS medium supplemented with 3 mg/L NAA to stimulate adventitious roots. The generated adventitious roots were grown on liquid MS medium to multiply biomass and used as raw materials to investigate the effects of some factors on the growth and accumulation of secondary compounds in adventitious roots (Fig. 1).

Figure 1: Diverse *in vitro* events of adventitious root culture of *Curculigo orchoides*; A. Samples of *Curculigo orchoides* leaves for 10 days after sterilization; B. Performance Modified MS +3 mg/L NAA; C. growth of adventitious roots in MS + 3 mg/L NAA liquid medium.



**Effect of Culture Conditions on Adventitious Root Growth of *Curculigo orchoides*:** The effect of MS strength, pH and sucrose concentration were evaluated in term of fresh weight, dry weight of adventitious roots produced for each treatment as shown in Table 1. pH affected the root development through an ion exchange process through its membrane. According to “developing hypotheses about the acid”, the size of cells were manipulated by its environment’s pH, where it decreases with decreased pH (Cosgrove, 1999). Winch and Pritchard (1999); Evans (1976); Edwards and Scott (1974) concluded that environmental pH induced root elongation. The effects of pH on the development of adventitious roots were proved by Link et al. (2009) while culturing *Orthosiphon stamineus* roots. pH 6.0 was the best among pH 4.0; 5.0; 5.8 và 7.0 and pH 6.0 was also appropriate for the adventitious roots development and saponin accumulation in *Panax ginseng* (Kim et al., 2005). The results showed that, the culture medium with pH = 6.5 is the best compared to the environment with pH under investigation for growth of *Curculigo orchoides* adventitious root (Fig 2.), with a mean value of 1.833 g FW and 0.147 g DW. Phenolics and Curculigoside content of *Curculigo orchoides* adventitious root were

not statistical difference in the culture medium with variable pH (Table 2).

Figure 2: Adventitious roots produced in different pH medium; (a) 5.5; (b) 6.0; (c) 6.5; (d) 7; (e) 7.5

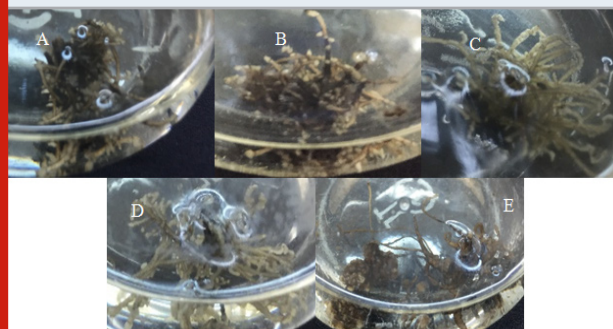
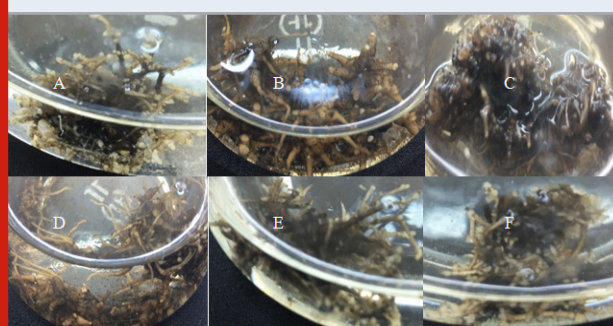


Figure 3: Adventitious roots produced in different sugar content; (a) 2%; (b) 3%; (c) 4%; (d) 5%; (e) 6%; (f) 7%.



Carbohydrate was essential for plant metabolism *In vitro*, therefore sugar content in the media had a crucial impact on the root induction and development. Sugar plays an important role in the regulation and expression of the transcription of photosynthesis genes (Sheen, 1990) and also to signals of abscisic acid and ethylene (Leon and Sheen, 2003). Cheng et al. (1992) reported sucrose concentration at 2-3 % positively affected the root induction of *Eucalyptus sideroxylon*. Moreover, sugar concentration was reported to have effects on adventitious roots induction of *Orthosiphon stamineus* and *Scopolia parviflora* at concentration 30% and 50%, respectively (Ling et al., 2009; Min, 2007). *Echinacea angustifolia* adventitious roots were cultured on bioreactor on MS media supplemented with 2 mg/l IBA and 50 g/L sucrose. The results showed that, the culture medium supplemented with 40 mg/L sucrose is the best compared to the environment with pH under investigation for growth of *Curculigo orchoides* adventitious root (Fig 3.), with a mean value of 2.471 g FW and 0.198 g DW. Moreover, the optimum sucrose concentration for highest production of secondary metabolites may be varied from every plant species (Koch, 2004).

The best sucrose concentration for production of phenolics, flavonoids and chlorogenic acid of *E. angustifolia* root suspension cultures was found to be 5% (w/v) (Wu at el., 2006) while for *Hypericum perforatum* adventitious root cultures, 5% to 9% (w/v)



of sucrose concentration demonstrated the increment of secondary metabolites production (Ganefianti et al., 2017). Similar observation also displayed in the present study of adventitious root culture of *Curculigo orchioides* where the highest curculigoside content (75.330 µg/treatment) was enhanced in MS medium supplemented with 4% (w/v) sucrose concentration (Table 2). These results were supported by the detection of curculigoside content through chromatogram peaks according to the sucrose concentrations treatments using HPLC (Fig. 5).

Figure 4: Adventitious roots produced in different strength of MS medium; (a) 1/4; (b) 1/2; (c) 3/4; (d) 1



These results may be due to the osmotic stress that occurred during the accumulation of high carbon ion in a nutrient medium that boost the production of secondary metabolite in plant cells (Cui et al., 2010; Praveen and Murthy, 2012; Wang and Weathers, 2007; Kusuma et al., 2016). In terms of nutrients requirement, different plants entail varying content of nutrients for growth. Based on Table 1, data recorded after four weeks of culture revealed that between all MS medium strengths, the fresh weight of adventitious roots cultured on the 3/4 strength of MS medium was found to produce the highest root biomasses with a mean value of 2.499 g FW and 0.200 g DW. Nutrients content has an important regulatory role on repressing the transcription of photosynthetic genes (Sheen et al., 1999) and interacting with abscisic acid and ethylene signaling (Yanagisawa et al., 2003). Medium salt strength also induced changes in growth, physiology and secondary metabolite content in adventitious roots of *Morinda citrifolia* (Baque et al., 2010).

*In vitro* adventitious root culture showed a high rate of proliferation and active secondary metabolism (Hahn et al., 2003; Yu et al., 2005). Therefore, a substantial increase in phenolic and curculigoside content was detected in the adventitious roots grown in modified MS medium that had the lower macro salt concentration. The results are in agreement with earlier studies on *Lobelia inflata* (Yonemitsu et al., 1990), *Fagopyrum esculentum* (Lee et al., 2007), and *Withania somnifera* (Murthy et al., 2008). Adventitious-root cultures of *Iris germanica* on liquid medium accumulated 4-5 fold higher contents of isoflavone, aglycones and glucosides in 3-week-old liquid cultures (Akashi et al., 2005). The

Table 1. The fresh weight and dry weight of adventitious root produced from each treatment with different MS strength, pHs and sucrose concentration after four weeks in culture.

Culture condition	Treatment	Fresh weight (FW) (g)	Dry weight (FW) (g)
pH	5.5	1.680 <sup>bc</sup>	0.134 <sup>bc</sup>
	6.0	1.766 <sup>ab</sup>	0.141 <sup>ab</sup>
	6.5	1.833 <sup>a</sup>	0.147 <sup>a</sup>
	7.0	1.718 <sup>bc</sup>	0.137 <sup>bc</sup>
	7.5	1.610 <sup>c</sup>	0.129 <sup>c</sup>
	CV	2.44	2.48
	LSD <sub>0.01</sub>	0.11	0.01
Sucrose concentration	2%	1.731 <sup>cd</sup>	0.138 <sup>cd</sup>
	3%	1.837 <sup>c</sup>	0.147 <sup>c</sup>
	4%	2.471 <sup>a</sup>	0.198 <sup>a</sup>
	5%	2.107 <sup>b</sup>	0.169 <sup>b</sup>
	6%	1.732 <sup>cd</sup>	0.139 <sup>cd</sup>
	7%	1.607 <sup>d</sup>	0.129 <sup>d</sup>
	CV	3.49	3.46
	LSD <sub>0.01</sub>	0.17	0.01
MS strength	1/4	1.877 <sup>d</sup>	0.150 <sup>c</sup>
	1/2	2.101 <sup>c</sup>	0.168 <sup>d</sup>
	3/4	2.499 <sup>a</sup>	0.200 <sup>a</sup>
	1	2.384 <sup>b</sup>	0.191 <sup>b</sup>
	CV	1.82	1.90
	LSD <sub>0.01</sub>	0.11	0.01

The average score with different letters are significantly different at p = 0.01 level.

z: Letters a, b, c, d in the same column represented the differences amongstreatments by t Tests (LSD).

present study is the first detailed description for testing different MS medium strengths for *In vitro* curculigoside production from adventitious root cultures. The produced curculigoside content were 76.521 (µg/Treatment) on the 3/4 strength of MS medium (Table 2).

Meanwhile, the root growth (fresh weight and dry weight of adventitious roots) showed a significant reduction in MS medium supplemented with 4% (w/v) sucrose. This may be due to higher osmotic pressure in the medium with the rising of sucrose concentration that gave deleterious effects to the root induction (Cui et al., 2010; Zhang et al., 2012; Tewtrakul et al., 2003). These could be observed through obvious adventitious root growth in Fig. 4. Therefore, it could be specified that reasonably low concentration of sucrose was suitable for adventitious roots growth whereas too low or too high sucrose concentration was not appropriate as it might not supply sufficient energy for root initiation which soon reduces the adventitious roots growth characteristics respectively.

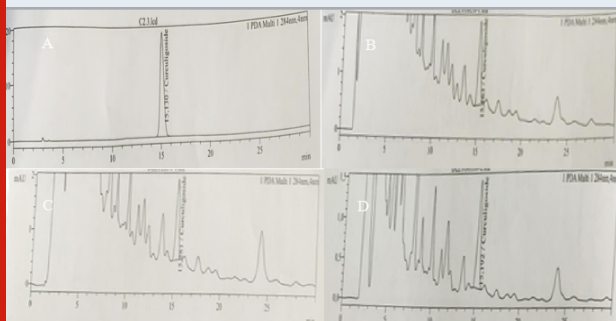
Table 2. The Phenolic and Curculigoside contents of adventitious root produced from each treatment with different MS strength, pHs and sucrose concentration after four weeks in culture.

Culture condition	Treatment	Concentration of Phenolic total (mg/g DW)	Concentration of Curculigoside (µg/g DW)	Concentration of Curculigoside (µg/Treatment)
pH	5.5	31.716 <sup>a</sup>	388.112 <sup>a</sup>	52.174 <sup>ab</sup>
	6.0	31.994 <sup>a</sup>	378.601 <sup>a</sup>	53.508 <sup>ab</sup>
	6.5	32.290 <sup>a</sup>	382.625 <sup>a</sup>	56.105 <sup>a</sup>
	7.0	31.650 <sup>a</sup>	390.017 <sup>a</sup>	53.587 <sup>ab</sup>
	7.5	31.575 <sup>a</sup>	377.512 <sup>a</sup>	48.640 <sup>b</sup>
	CV	2.83	1.95	3.83
	LSD <sub>0.01</sub>	2.34	19.35	5.24
Sucrose concentration	2%	32.018 <sup>a</sup>	381.134 <sup>a</sup>	52.769 <sup>cd</sup>
	3%	32.865 <sup>a</sup>	382.405 <sup>a</sup>	56.187 <sup>c</sup>
	4%	30.435 <sup>b</sup>	381.013 <sup>a</sup>	75.330 <sup>a</sup>
	5%	31.823 <sup>a</sup>	380.848 <sup>a</sup>	64.200 <sup>b</sup>
	6%	31.993 <sup>a</sup>	382.111 <sup>a</sup>	52.937 <sup>cd</sup>
	7%	31.833 <sup>a</sup>	382.677 <sup>a</sup>	49.186 <sup>d</sup>
	CV	1.61	0.47	3.91
LSD <sub>0.01</sub>	1.27	4.44	5.70	
MS strength	1/4	29.036 <sup>c</sup>	367.849 <sup>b</sup>	55.229 <sup>d</sup>
	1/2	29.667 <sup>bc</sup>	377.046 <sup>a</sup>	63.364 <sup>c</sup>
	3/4	31.041 <sup>ab</sup>	382.767 <sup>a</sup>	76.521 <sup>a</sup>
	1	31.849 <sup>a</sup>	383.870 <sup>a</sup>	73.201 <sup>b</sup>
	CV	1.85	0.88	1.72
	LSD <sub>0.01</sub>	1.54	9.11	3.15

The average score with different letters are significantly different at p = 0.01 level.

z: Letters a, b, c, d in the same column represented the differences among treatments by t Tests (LSD).

Figure 5: HPLC chromatogram for curculigoside content in adventitious roots of *Curculigo orchoides* cultured on (A) Standart, (B) pH 6.5, (C) 4% sucrose, (D) 3/4 strength MS medium.



## CONCLUSION

HPLC analysis displayed the presence of curculigoside from the *in vitro* adventitious roots of *Curculigo orchoides*. The growth characteristics (biomass) together with phenolic and curculigoside content

from the established *in vitro* adventitious roots of *Curculigo orchoides* was successfully enhanced by the manipulation of culture conditions involving 3/4 strength of MS medium and the addition of 4% (w/v) of sucrose. Hence, it is important to study other strategies in order to enhance the maximum production of secondary metabolites in *in vitro* systems.

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