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Full Length Research Paper

A simple and efficient procedure to enhance artemisinin content in *Artemisia annua* L. by seeding to salinity stress

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Artemisinin is an effective anti-malarial drug extracted from *Artemisia annua* L. Due to the low content of artemisinin in *A. annua*, great efforts have been devoted to improve artemisinin production. Here we report a simple and efficient procedure to enhance artemisinin content in *A. annua* by seeding to salinity stress. Our result shows that artemisinin content in the plant treated with 4 - 6 g/l NaCl could be significantly enhanced (up to 2 - 3% dry weight) compared to that in control plant (1% dry weight).

Key words: *Artemisia annua* L., artemisinin, NaCl, salinity stress.

INTRODUCTION

As one of the world's most serious parasitic diseases, malaria, caused by *Plasmodium*, causes at least 500 million cases globally every year, resulting in more than one million deaths (Snow et al., 1999; Ro et al., 2006). The biggest challenge facing in the fighting against malaria is the multi-drug resistance of *Plasmodium* strains to the widely used antimalarials such as chloroquine, mefloquine and sulfadoxine-pyrimethamine (Greenwood and Mutabingwa, 2002; Liu et al., 2006).

Artemisinin, an endoperoxide-containing sesquiterpene isolated from *Artemisia annua* L., an annual herb native to China, is becoming more and more popular in treating malaria. Nowadays, the toxin-free, artemisinin-based combination therapy (ACT) is recommended by World Health Organization (WHO) as the most effective against the drug-resistant malaria (Olliaro and Taylor, 2004; Shetty et al., 2004; Davis et al., 2005).

Artemisinin content in *A. annua* is very low (0.01 - 1% dry weight, DW), and the demand for artemisinin is increasing along with the increasing number of people suffering from malaria. Various approaches have been attempted to increase artemisinin production including chemical synthesis (Xu et al., 1986; Avery et al., 1992) and genetic engineering of the pathway genes involved in artemisinin biosynthesis in *A. annua* (Vergauwe et al., 1996; Chen et al., 2000; Xie et al., 2001; Martin et al., 2003; Ro DK et al., 2006), but not much success has been recorded because of the high cost or complex nature of the gene regulation and expression in artemisinin biosynthesis. New approaches, cheaper and more convenient, are needed for improving artemisinin production.

Since artemisinin is a kind of secondary metabolites (Aharoni et al., 2005; Jorgensen et al., 2005), climatic condition together with the way and time of planting and harvesting of *A. annua* can influence artemisinin production in *A. annua* (Wallaart et al., 2000; Ram et al., 1997). Plant hormone such as GA_3, BA and kinetin may also influence artemisinin production (Whipkey et al., 1992; Fulzele et al. 1995; Gulati et al., 1996; Smith et al., 1997; Weathers et al., 2005). In addition, stress conditions such as light, temperature and watering may have effects on artemisinin production too (Wang et al., 2001; Liu et al., 2002; Wang et al., 2002; Guo et al., 2004; Wallaart et al., 2000).

In this study, we tried to investigate if salinity, one of the commonest stresses, could influence artemisinin pro-

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duction in *A. annua* and our result demonstrated that by simply treating *A. annua* with NaCl, the content of artemisinin in the plant could be significantly enhanced.

MATERIALS AND METHODS

Plant material, growth condition and salinity treatments

A. annua L. was collected from Sichuan Province, China. Seeds of *A. annua* were surface sterilized with 20% (v/v) sodium hypochlorite (NaOCl) for 20 min, sown onto germination medium (Murashige and Skoog, 1962) basal medium with the addendum of sucrose (30 g/l) and phytagel (Sigma) (2.6 g/l) in a growth chamber with a photoperiod of 16 h light\8 h dark and light intensity of 80,000 Lux (metal halide source) at 26ºC and grown for 4 weeks.

When about 5 cm in height, the geminated seedlings were transplanted into plastic pots containing peat-moss and perlite at the ratio of 1:1. To adjust to the new surrounding, the plants were watered by normal nutrient solution (33 mg/l NH_4NO_3, 38 mg/l KNO_3, 8.8 mg/l $CaCl_2{\cdot}2H_2O$, 7.4 mg/l $MgSO_4{\cdot}7H_2O$, 3.4 mg/l KH_2PO_4) in the first two weeks, then treated with the normal nutrient solution containing different concentrations of NaCl (0 as the control, 2, 4, 6 and 8 g/l) respectively. The plants were watered with nutrient solution containing NaCl every 2 d. Five individual plants in each level of salinity treatment were selected randomly for artemisinin quantification from 26-35 plants treated for each level. The experiments were replicated 2 times. The contents of artemisinin in the treated plants together with the un-treated control plants were measured by High Performance Liquid Chromatography (HPLC) before the flowering of the plants.

Sample preparation

Artemisinin in the plants was extracted based on the method of Van Nieuwerburgh et al. (2006). Fresh leaves of *A. annua* (1-2 g fresh weight) were immersed in 10 ml chloroform in 50 ml eppendorf tube and mixed by gentle shaking for 1 min. Then the solution was moved to another fresh tube to volatilize chloroform, and then 3 ml ethanol was added into the tube to dissolve the extract. The leaf material after the extraction was weighed (to measure the dry weight) after dried at 60ºC for 4 h.

Samples were pre-column derivatised for HPLC. The extract dissolved in ethanol (100 l) was mixed with 400 l 0.05M NaOH, and then heated at 50ºC for 30 min. After cooling, 500 l 0.08 M HAc was added for quantification with HPLC.

Quantification of artemisinin with HPLC

The samples were analyzed by Waters Alliance 2695 HPLC system. The mobile phase (pump A, methanol; pump B, 0.01M HAc/NaAc buffer adjusted to pH 5.8 with acetic acid) for gradient elution was eluted at a flow rate of 1 ml/min. The initial composition of 50%:50% (v/v) was maintained for 2 min, then, the methanol content was increased linearly to 70% over a period of 4 min and maintained for 2 min. The column was re-equilibrated for 10 min between runs. The injection volume was 10 l for all samples, and the results were analyzed using Waters 2996 PDA wavelength detector and Empower data system with UV detection of peaks at 260 nm. The artemisinin purchased from Sigma was used as the standard control in the measurement.

RESULTS AND DISCUSSION

The content of artemisinin in *A. annua* under salinity stress

We determined the artemisinin contents in *A. annua* L. plants seeding to 5 levels of NaCl salinity stresses with HPLC. For each level we treated 26 - 35 individual plants and repeated the experiment twice. We found that the plants seeding to salinity stress had higher contents of artemisinin (2 - 3% DW) compared to those without treatment (1.0 - 1.5% DW) (Figure 1). The result analyzed with two-side T test suggested that the enhancement of artemisinin content caused by 2 g/l NaCl stress was not significant compared to the control, but the enhancement caused by 4 and 6 g/l NaCl stresses was extremely significant ($P<0.01$) compared to the control (Figure 1).

Various approaches have been previously tried to enhance artemisinin production (Vergauwe et al., 1996; Chen et al., 2000; Wang et al., 2001; Xie et al., 2001; Liu et al., 2002). Wang et al. (2001) found that light spectrum would influence biomass and artemisinin content of transformed hairy roots. The highest biomass (5.73 g DW/l) and artemisinin content (31 mg/g) were obtained under red light at 660 nm which were 17 and 67% higher than those obtained under white light, respectively. Liu et al. (2002) found that light irradiation influenced the growth and production of artemisinin in transformed hairy root cultures of *A. annua* too. When hairy roots were cultured under illumination of 3,000 Lux for 16 h using several cool-white fluorescent lamps, the dry weight and artemisinin concentration reached 13.8 and 244.5 mg/l, respectively. Wang et al. (2002) regulated the ratio of NO_3/NH_4 and total initial nitrogen concentration in the culture of hairy roots, and successfully increased artemisinin concentration by 57% compared to the control.

Xie et al. (2001) infected *A. annua* leaf pieces and petiole segments with *A. rhizogenes* and obtained a clone of hairy root with artemisinin content of 0.12% DW. Vergauwe et al. (1996) transformed *A. annua* plants mediated by *Agrobacterium tumefaciens* and slightly higher artemisinin content (0.17% DW) in the leaves of regenerated plant than normally cultured plant (0.11% DW) was achieved. Chen et al. (2000) transformed a cDNA encoding cotton FDS (farnesyl diphosphate synthase) under the control of CaMV 35S promoter into *A. annua* via *A. tumefaciens* or *A. rhizogenes*. By over-expressing FDS, a key enzyme in the biosynthesis of artemisinin, in transgenic plants, the con-tent of artemisinin reached approximately 0.8 - 1% DW.

In this study, the artemisinin content in *A. annua* plants could reach 2 - 3% DW, the highest artemisinin content in *A. annua* reported so far, by the procedure of treating plants with suitable concentrations of NaCl.

The influence of salinity stress on the growth of *A. annua* plants

To investigate if the salinity treating procedure which could elevate the arteminsinin content in *A. annua* could influence the growth of *A. annua*, we measured the growth of the plants treated with different NaCl concentrations (2, 4, 6 g/L) and compared to that of the normal

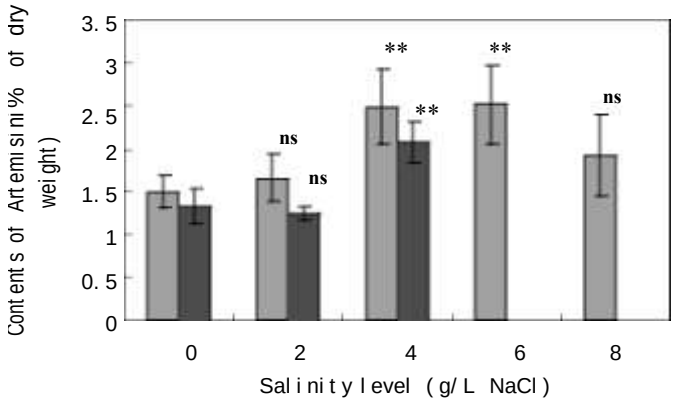

Figure 1. Contents of artemisinin in *A. annua* plants under salinity treatments. The results were analyzed with a two-side T test on the artemisinin contents in the plants under salinity stress compared to the control (ns: P>0.05; **: P<0.01) . Data represent the means ± SE (standard errors, vertical bars) of 5 randomly selected individual plants from each NaCl treatment after 1 month. The dark columns with 0, 2 and 4 g/L NaCl were average artemisinin contents of the plants in the repeated experiment; another 5 randomly individual plants were selected from each NaCl treatment. The data were obtained after 3 months of NaCl treatment. The plants could not survive after 3 months of 6 or 8 g/L NaCl treatment, so there were no artemisinin data.

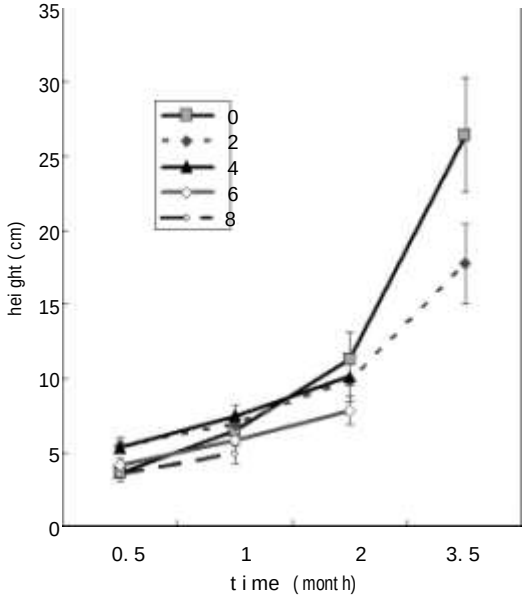

Figure 3. The height (h) of *A. annua* plants seeding to different t levels of salinity stress (0: 0 g/l NaCl; 2: 2 g/l NaCl; 4: 4 g/l NaCl; 6: 6 g/l NaCl; 8: 8 g/l). Data represent the means ± SE (standard errors, vertical bars) of 26 to 35 individual plants.

untreated ones (0 g/l NaCl). The result showed that low levels of salinity stress (2 and 4 g/l NaCl) did not influence much the growth of *A. annua* plants at first 2 months of the treatment (Figure 2A-C), but slowed down

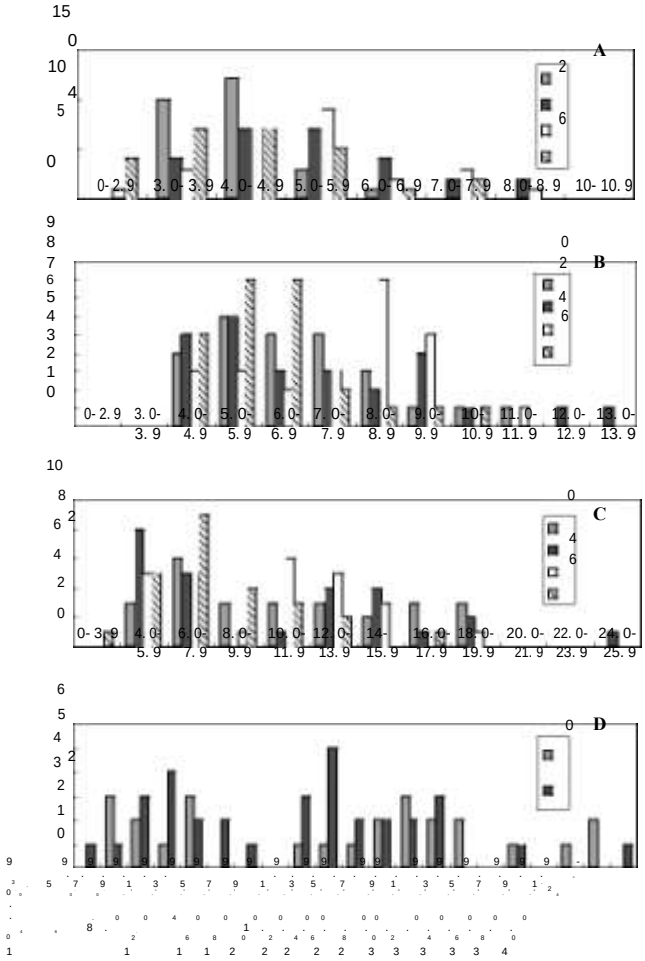

Figure 2. The distribution for the height (cm) of individual plants of *A. annua* under the different levels of salinity stress (0: 0 g/l NaCl; 2: 2 g/l NaCl; 4: 4 g/l NaCl; 6: 6 g/l NaCl) in four growth stages (A: 0.5 month; B: 1 month; C: 2 months; D: 3.5 months).

the growth of the plants later on (Figure 2D). Higher level of salinity stress (6 g/L NaCl) slowed down the growth of the plants from the beginning of the treatment (Figure 2A-C). Similar result was also obtained when summarizing the average height of plants seeding to different salinity stresses (Figure 3).

Conclusion

From the present study, it is obvious that salinity stress at current NaCl concentrations inhibit the development of *A. annua* plants, and influences the contents of artemisinin in plants. It is important to find a more proper salinity stress and treating occasion and time which do not hamper much the growth of the plants but enhance the accumulation of artemisinin in plants to high level. It was reported earlier that the contents of artemisinin in *A. annua* plants varied in different plant developmental stages, and reached to the apex before flowering or in the full flowering period (Abdin et al., 2003). Based on the

previous observations and the results from the present study, it is speculated that it is possible to get the maximal artemisinin accumulation in *A. annua* plants via treating the plants with a salinity stress of 2 - 4 g/L NaCl for about one or two months just before flowering of the plants. By doing this, the influence of salinity stress to the growth of the plants may be greatly reduced but the accumulation of artemisinin in plants could be significantly enhanced. This study will be carried out in the future.

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REFERENCES

Abdin MZ, Israr M, Rehman RU, Jsin SK (2003). Artemisinin a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Med. 69: 289-299.

Aharoni A, Jongsma MA, Bouwmeester HJ (2005). Volatile science Metabolic engineering of terpenoids in plants. Trends Plant Sci. 10: 594-602.

Avery MA, Chong WKM, Jennings-White C (1992). Stereoselective total synthesis of (+)-artemisinin the antimalarial constituent of Artemisia annua L J. Am. Chem. Soc. 114: 974-979.

Chen DH, Ye HC, Li GF (2000). Expression of a chimeric farnesyl diphosphate synthase gene in Artemisia annua L. transgenic plants via Agrobacterium tumefaciens-mediated transformation. Plant Sci. 155: 179-185.

Davis TM, Karunajeewa HA, Ilett KF (2005). Artemisinin-based combination therapies for uncomplicated malaria. Med. J. Aust. 182: 181-185.

Fulzele DP, Heble MR, Rao PS (1995). Production of terpenoid from Artemisia annua L. plantlet cultures in bioreactor. J. Biotechnol. 40: 139-143.

Greenwood B, Mutabingwa T (2002). Malaria in 2002. Nature 415: 670-672.

Gulati A, Bharel S, Jain SK, Abdin MZ, Srivastava PS (1996). In vitro micropropagation and flowering in Artemisia annua. J. Plant Biochem. Biotech. 5: 31-35.

Guo C, Liu CZ, Ye HC, Li GF (2004). Effect of temperature on growth and artemisinin biosynthesis in hairy root cultures of Artemisia annua. Acta Bot. Boreal-Occident Sin. 24: 1828-1831.

Jorgensen K, Rasmussen AV, Morant M, Nielsen AH, Bjarnholt N, Zagrobelny M, Bak S, Moller BL (2005). Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr. Opin. Plant Biol. 8: 280-291.

Liu C, Zhao Y, Wang Y (2006). Artemisinin: current state and perspectives for biotechnological production of an antimalarial drug. Appl. Microbiol. Biotechnol. 72: 11-20.

Liu CZ, Guo C, Wang YC, Ouyang F (2002). Effect of light irradiation on hairy root growth and artemisinin biosynthesis of Artemisia annua L. Process Biochem. 38: 581-585.

Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003). Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. Nat. Biotechnol. 21: 796-802.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Olliaro PL, Taylor WR (2004). Developing artemisinin based drug combinations for the treatment of drug resistant falciparum malaria: A review. J. Postgrad. Med. 50: 40-44.

Ram M, Gupta MM, Dwivedi S, Kumar S (1997). Effect of plant density on the yields of artemisinin and essential oil in Artemisia annua cropped under low input cost management in north-central India. Planta Med. 63: 372-374.

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440: 940-943.

Shetty P (2004). Global fund switches to artemisinin. Lancet Infect. Dis. 4: 477.

Smith TC, Weathers PJ, Cheetham RC (1997). Effect of gibberellic acid on hair root cultures of Artemisia annua growth and artemisinin production. In vitro Cell. Dev. Biol. Plant 33: 75-79.

Snow RW, Craig M, Deichmann U, Marsh K (1999). Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull. World Health Organ. 77: 624-640.

Van Nieuwerburgh FC, Vande Casteele SR, Maes L, Goossens A, Inze D, Van Bocxlaer J, Deforce DL (2006). Quantitation of artemisinin and its biosynthetic precursors in Artemisia annua L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. J. Chromatogr. A 1118: 180-187.

Vergauwe A, Cammaert R, Vandenberghe D, Genetello C, Van Montagu M, Van den Eeckhout E (1996). Agrobacterium tumefaciens-mediated transformation of Artemisia annua L.and regeneration of transgenic plant. Plant Cell Rep. 15: 929-937.

Wallaart TE, Pras N, Beekman AC, Quax WJ (2000). Seasonal variation of artemisinin and its biosynthetic precursors in plants of Artemisia annua of different geographical origin: proof for the existence of chemotypes. Planta Med. 66: 57-62.

Wang JW, Tan RX (2002). Artemisinin production in Artemisia annua hair root cultures with improved growth by altering the nitrogen source in the medium. Biotechnol. Lett. 24: 1153-1156.

Wang YC, Zhang HX, Zhao B, Yuan XF (2001). Improved growth of Artemisia annua L. hairy roots and artemisinin production under red light conditions Biotechnol. Lett. 23: 1971-1973.

Weathers PJ, Bunk G, Mccoy MC (2005). The effect of phytohormones on growth and artemisinin production in Artemisia annua hairy roots. In Vitro Cell. Dev. Biol. Plant. 41: 47-53

Whipkey A, Simon JE, Charles DJ, Janick J (1992). *In vitro* production of artemisinin from Artemisia annua L. Phytother. Res. 1: 15-25.

Xie DY, Zou ZR, Ye HC, Li GF, Guo ZC (2001). Selection of hairy root clones of Artemisia annua L. for artemisinin production. Israel J. Plant Sci. 49: 129-134.

Xu XX, Zhu J, Huang DZ, Zhou WS (1986). Total synthesis of arteannuin and deoxyarteannuin. Tetrahedron 42: 819-828.