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Elicitation of pharmacologically active phenolic compounds from *Rauvolfia serpentina* Benth. Ex. Kurtz.

Vadakkemuriyil Divya Nair[a], Rajaram Panneerselvam[a], Ragupathi Gopi[a,*], Shao Hong-bo[b]

[a] *Stress Physiology Lab, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu 608002, India*
[b] *Institute of Life Sciences, Zheng Zhou Road-53, Quindao University, University of Science and Technology, Quindao 266042, China*

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ABSTRACT

Quality of medicinal plants used for the production of galenics or pharmacologically useful compounds is usually assessed by the content of biologically active compounds. Most of these plants are grown in fields, this study focused to evaluate the effect of three natural elicitors salicylic acid, methyl salicylate and acetyl salicylic acid (SA, MSA, ASA) on phenolic compound content of *Rauvolfia serpentina*, and to compare the efficiency of several solvent system in phenolic compound extraction from this plant. A tremendous increase of phenolics and stimulation of the biomass yield were achieved. Tuning of organ specificity by modification of the concentration of elicitor was also observed. This methodology represents a convenient alternative to cell suspension or hydroponic cultures being applicable in wide agricultural practice and to improve phenolic concentration during extraction.

1. Introduction

Plants contain a large variety of phytonutrients many having antioxidant properties. Antioxidant compounds include vitamins, phenols carotenoids and flavonoids. Phenolics are secondary plant metabolites found in the majority of herbs, vegetables, and tea (Bonilla et al., 2003). In recent years many studies have demonstrated that free radicals are the leading cause of degenerative disease (Bonilla et al., 2003). Plant antioxidants work as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers and enzyme inhibitors (Wang and Lin, 2000). Many of these protective biological effect are attributed to polyphenol contents of plants (Bouayed, 2010). There is interest in knowing the phenolic content of medicinal plants in order to increase their potential use as neutraceutical or as source of natural antioxidants in food and cosmetic industry (Bonilla et al., 2003). Synthetic antioxidants such as butylated hydroxyl toluene (BHT) butylated hydroxyl anisole (BHA) and propyl gallate (PG) have been widely used as antioxidants in the food industry (Nawar, 1996). However the safety of these synthetic antioxidants has been questioned. BHA has shown to be carcinogenic and BHT has been related to internal and external hemorrhaging at high doses in rats and Guinea pigs (Ito et al., 1983). These findings together with consumer interest in natural food additives have reinforced the need for effective antioxidants from natural resources as an alternative to prevent deterioration of food during processing and storage. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as related lipid oxidative rancidity in food, cosmetics, and pharmaceutical materials (Lai et al., 2001).

Phenolic acids form a diverse group that includes the widely distributed hydroxycinnamic acids, most frequently as simple esters with hydroxycarboxylic acid or glucose, and hydroxybenzoic acid compounds which are present mainly in the form of glucosides. The presence of the —CH=CH—COOH group in hydroxycinnamic acids is considered to be key for the significantly higher antioxidative efficiency than the —COOH in the hydroxybenzoic acids (White and Xing, 1997). The main source of these compounds is several types of plant material such as vegetables, fruits, leaves, oilseeds, cereal crops, spices, and herbs (Ramarathnam et al., 1997). In recent years researchers and food manufacturers have become increasingly interested in these compounds, which may be exploited for the development of health foods or nutraceuticals (Truswell, 2003).

The elicitors are signals triggering the formation of secondary metabolites. Use of elicitors of plant defense mechanism i.e. elicitation have been one of the most effective strategy for improving the productivity of bioactive secondary metabolites. Production of many variable secondary metabolites using various elicitors was reported (Wang and Zhong, 2002; Hu et al., 2008; Hiraoka et al., 2004; Giri et al., 2012). Addition of elicitors (which could be of biotic or abiotic origin) to the nutrient medium is commonly exploited in plant cell cultures. Recently the stimulation of plants by addition of elicitors to hydroponic media has also been investigated. However, despite the fact that the majority of medical plants are currently grown in field conditions, very little research has focused

* Corresponding author. Tel.: +91 9443669384.
E-mail address: suriyagopi@gmail.com (R. Gopi).

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on elicitation of plants grown on soil to improve the production of pharmaceutically active substances.

In our attempts to develop useful technology for large scale production of plant secondary metabolites, we suggested that the application of certain stress hormones on the whole intact plants can increase the content of secondary metabolites (Jaleel et al., 2009a,b). A similar approach was successfully used by other groups for effective stimulation of red clover (*Trifolum pretense* L.) (Sivesind and Seguin, 2006) as well as phenolics in black chokeberry (*Aronia melanocarpa* (Michx) Elliot) (Hudec et al., 2006), black currant (*Ribes nigrum* L.) (Hudec et al., 2009), nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) (Hudec et al., 2007).

Salicylic acid (SA), a plant hormone, plays an important role in abiotic stress tolerance (Pooja and Sharma, 2010). Convincing data have been obtained concerning the salicylic acid increase the resistance in bean, tomato, desert pea, corn, wheat, maize, and rice to salinity (Afran et al., 2007; Gunes et al., 2007; Senaratna et al., 2007).

Numerous reports indicated that the amount of phenolic compounds varied with conditions of extraction. Solvent systems have been shown to have different extraction ability and there is no uniform solvent system for phenolic compound extraction (Rickert et al., 2004; Kao et al., 2004). The more efficient solvent system for the extraction of phenolics needs to be investigated to better differentiate among samples.

Rauvolfia serpentina Benth. Ex. Kurtz. is a genus of the family Apocyanaceae (dogbane family) sub-family Plumeroidae and occurs in nearly all habitable tropical and subtropical regions. *R. serpentina* is important from a medicinal point of view because of the presence of N-containing indole alkaloids, which are localized in roots (Hu et al., 2008). Reserpine is most prominent of these alkaloids and is useful in the treatment of hypertension, cardiovascular diseases, nervous disorders and as a tranquilizing agent that is in great demand by modern pharmaceutical industries (Weiss and Fintlemann, 2000).

Objectives of this study were to evaluate the effects of three natural elicitors, salicylic acid, and its derivatives methyl salicylate, and acetyl salicylic acid at various concentrations on phenolic acid content in *R. serpentina* and to compare the efficiency of solvent systems frequently used in phenolic acid extraction and quantification.

2. Materials and methods

2.1. Plant cultivation and elicitor treatment

Plants were grown in the botanical garden, department of Botany, Annalamalai University. Seedlings were raised from root cuttings on seed beds. Nursery beds were watered regularly for the healthy growth of seedlings. After one month, seedlings were transplanted to twelve cement vials having 1 m diameter with twelve plants in each vial for experimental alternative. Prior to transplantation the vials were filled with red soil, sand and farmyard manure (FYM) in a 1:1:1 ratio. Vials were irrigated immediately after transplantation and subsequent irrigation was done twice in a day to keep the optimum moisture level required in the soil. Samples were randomly selected from the treated and control plants for further analysis.

All elicitors were dissolved in distilled water and these solutions were sprayed directly on plants (100 mL per plant). Acetyl salicylic acid (ASA), salicylic acid (SA) and methylsalicylic acid (MS) were applied in three different concentrations (10, 100 and 1000 μM). Water was sprayed as a negative control.

Plants in three cement vials were taken for each treatment with different elicitors at different concentrations and the other three were kept untreated and served as control. Elicitor treatment was given to each plant as foliar spray. The treatment was given with 20 days' interval on 50th, 70th and 90th days after planting (DAP). Plants were harvested, on 100th DAP, root and shoot separately.

2.2. Analysis of plant material

2.2.1. Shoot and root fresh and dry weight

After washing the plants in tap water, the fresh weight was determined by using an electronic balance and the values were expressed in grams. After that, plants were dried at 60 °C in a hot air oven for 48 h. After drying, the weight was measured and the values were expressed in grams.

2.2.2. Extraction of phenolic compounds

Ten grams of shade dried plant material (shoot and root) from each plot was ground in a Knifetec 1095 smple mill (Foss, Inc., Eden Prairie, MN) and passed through a 60 mesh sieve (W.S. Tyler Inc., Mentor, OH). Eight solvent systems were used to extract phenolic compounds: 53%AcCN/H_2O, AcCN/H_2O/0.1 N hydrochloric acid HCl (10:7:2), 53% methanol (MeOH)/H_2O, MeOH/H_2O/0.1NHCl (10:7:2), 53% ethanol (EtOH)/H_2O, EtOH/H_2O/0.1 N HCl(10:7:2) and H_2O at 60 °C and ambient temperature. A total of 0.2 g of flour of each sample and 2 mL of solvent were mixed and shaken at 250 rpm for 2 h at ambient temperature and then centrifuged at 7000 × *g* for 5 min.The supernatant was used for phenolic compound analysis.

2.2.3. Phenolic compound analysis

After the supernatant had been filtered through a 0.2 μm PVDF Targeted Syringe Filter (National Scientific, Duluth, GA), 6 μL of filtrate was injected into the cartridge during HPLC for phenolic compound analysis. Each sample was extracted thrice separately and evaluated independently by HPLC for six phenolic compounds (Fig. 1). A model 1090 Hewlett–Packard (Avondale, PA) liquid chromatograph equipped with a diode array ultraviolet (UV) detector was used to analyze phenolic compound content. A TSK GEL Super – ODS column (Supelco, Bellefonte, PA) was used with a flow rate of 1.0 mL/min at 37 °C. The effluent absorbance at 254 nm was used to detect the separated phenolic compounds. The mobile phase consisted of two solvent systems, 0.1% trifluoroacetic acid in acetonitrile (solvent system A) and 0.1% trifluoroacetic acid in Millipore water (solvent system B). Solvent system B was set up at 100% at the initial running, then changed to 50% during the first 30 min, and then returned to 100% within the last 5 min. Six microliters of each sample was injected in for phenolic compound quantification according to the phenolic compound standard curve. Each sample was extracted and injected in triplicate.

2.2.4. Chemicals and reagents

All the standards and chemicals were purchased from Sigma–Aldrich Ltd. (St. Louis, MO, USA) and we used without additional purification. All the reagents used were of analytical grade except those for HPLC, which were of HPLC grade and also were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2.5. Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Values are mean ± SD for six samples in each group. *P* values ≤0.05 were considered as significant.