Effect of methyl jasmonate and salicylic acid on noradrenalin accumulation in hairy roots of \textit{Portulaca oleracea} L.

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ABSTRACT: \textit{Portulaca oleracea} L. is a medicinal plant find in Europe and Asia. This plant contains valuable secondary metabolite such as noradrenalin. Naturally the wild plant produce small amount of this secondary metabolites so induction of hairy roots can be useful to increase its production. In our research \textit{Agrobacterium rhizogenes} strain AR15834 was used to induce hairy roots in the two-week leaf explants of \textit{P. oleracea}. PCR analysis with specific primers rolB gene was performed to confirm the transgenic hairy roots production. PCR analysis results showed diagnostic bands with size 780 bp related to specific reproduction of rolB gene, so the transgenic hairy roots was confirmed. The effect of two chemical elicitors, salicylic acid and methyl jasmonate, on the production of noradrenalin in \textit{P. oleracea} hairy roots was examined. Methyl jasmonate was found to increase the production of noradrenalin. The optimal methyl jasmonate dose was between 100um and 200um for hairy roots harvested 48h after elicitation. After 48h of induction with 200um methyl jasmonate, an eightfold increase in the level of noradrenalin was observed in compared with control cultures.

Keywords: \textit{Portulaca oleracea}, Noradrenalin, hairy root, \textit{A. rhizogenes}, PCR, Salicylic, Methyl jasmonate

Introduction

Common purslane (\textit{Portulaca oleracea} L.), which is a member of \textit{Portulacaceae}, is widespread as a weed and has been ranked the eight most common plant in the world (Liu et al., 2000). It has the abilities of antibacterial, -virus, -antherasis, -caducity, -diabetes, and enhancing immunity (Zheng et el. 1997). Recent studies indicated that the consumption of \textit{Portulaca oleracea} may help to reduce the occurrence of cancer and heart diseases (Simpoulos and Clin, 1991). \textit{Portulaca oleracea} is a rich source of omega-3 fatty acids, gallotannins, kaempferol, quercetin and apigenin and contains abundant catecholamines, noradrenalin (NA) and dopamine (DA) that were demonstrated to be the major bioactive constituents (Simpoulos, 2004).

Noradrenalin or norepinephrine (abbreviated NA or NAD) is a catecholamine with multiple roles including a hormone and a neurotransmitter (Lewis, 2003). As a stress hormone, noradrenalin affects parts of the brain where attention and responding actions are controlled. Also, it underlies the fight-or-flight response, directly increasing heart rate, triggering the release of glucose from energy stores, and increasing blood flow to skeletal muscle. Noradrenalin can suppress neuroinflammation when released diffusely in the brain from the locus ceruleus (Heneka et al. 2010). When noradrenalin acts as a drug it increases blood pressure by increasing vascular tone through $\alpha$-adrenergic receptor activation. The resulting increase in vascular resistance triggers a compensatory reflex that overcomes its direct stimulatory effects on the heart, called the baroreceptor reflex, which results in a drop in heart rate called reflex bradycardia. Also, some studies show NA is a modulator of the immune system postulated to have
anti-cancer properties (Cook-Mills et al. 1995). But these commercially valuable phytochemicals are secondary metabolites that are produced in small amounts, and often accumulate in specialized tissues. A new method for enhancing noradrenalin production is by transformation using the natural vector system *Agrobacterium rhizogenes*, the causative agent of hairy root disease in plant. Hairy roots are formed when plant tissues are inoculated with *A. rhizogenes*, a gram-negative soil bacterium. The plant tissues are transformed by the Ri (root inducing) plasmid from the bacteria, causing genetically stable adventitious roots to grow from the infection sites, which can be excised and grown independently on hormone-free solid and liquid medium containing sugar, a suitable nitrogen source, and appropriate minerals and vitamins (Guillon et al. 2006). The fast growing hairy roots are unique in their genetic and biosynthetic stability and their fast growth can be used as a continuous source for the production of valuable secondary metabolites. Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further subculturing and plant regeneration.

Enhancement of secondary metabolites by elicitation is one of the few strategies which have recently found commercial application. Elicitors are compounds of mainly microbial origin or non-biological origin, which upon contact with higher plant cells; trigger the increased production of pigments, flavones, phytoalexins and other defense related compounds (Singh, 1999). In general, the secondary metabolites that are involved in plant defense functions undergo significant elicitation as a response to external physical, chemical and biological stimuli. A combination of inappropriate medium and elicitor as well as unsuitable concentration of the latter can result in ineffective elicitation. For example, phenyl propanoid (PP) pathway was not induced in all cultures of Vanilla planifolia by yeast extract (Funk and Brodelius, 1990), indicating that a successful elicitation is a very challenging process requiring intense screening procedures.

A biotic Elicitor molecules such as salicylic acid (SA) and methyl jasmonate (MJ) are known to induce the production of secondary metabolites when added to culture medium (Shanks and Morgan 1999) and promote the production of terpenes (Penuelas et al. 2007) including taxol (Yuan et al. 2002; Wang et al. 2004). Because noradrenalin is present in *Purtolaca oleracea* hairy roots, we hypothesized that elicitor-increase secondary metabolite production in *Purtolaca oleracea* hairy roots. In this research, the effect of two commonly used phytochemical elicitors, SA and MeJ, on the production of Noradrenalin in a *purtolaca oleracea* hairy root culture line was studied. In addition, we determined the optimum dosage of the elicitors, on the production of Noradrenalin in a *purtolaca oleracea* hairy root culture line.

**Materials and Methods**

**Plant material**

*P. oleracea* seeds were obtained from bu-Ali Sina garden, Hamedan, Iran. The seeds were washed with sterile distilled water and were surface-sterilized in 70% ethanol for 1min. After being washed three times with sterilized water, the seeds were immersed in 2% sodium hypochlorite for 1min and then germinated on hormone-free 1/2 Murashige and Skoog (1/2MS) medium containing 15g sucrose, pH 5.8. Germination started within 3 d and was carried out at (25 ± 1) °C under 16 h light/day. Plantlets were used for hairy roots induction.

**Hairy root culture**

*P. oleracea* hairy roots induced by (AR15834) were used as hairy roots source. For induction of hairy root, the 14 day old leaf explants immersed in liquid LB medium for 10 min. After 10 min for cocultivation with *A. rhizogenes*, explants were cultured on ½ MS medium at 25°C for 48h and then for elimination of *A. rhizogenes*, transferred to ½ MS medium with 300 mg/l CF. At 2-week intervals, infected segments were transferred to ½ MS medium with 200 mg/l and 100 mg/l CF, respectively. After many times of subcultures and complete elimination of bacteria from hairy roots for their molecular analyze, using the CTAB method (Cai et al. 1997), for extraction of DNA and PCR primers were used for amplification of a 780 bp fragment of the rolB gene in T-DNA entering to nuclear explants genome (Dhakulkar et al. 2005).

**Elicitation**

In this study, we used methyl jasmonate (MJ, purchased from Sigma Aldrich, 95% purity) in concentrations of 0(control), 50, 100, 200 μl and salicylic acid (SA, purchased from Merck) in concentrations of 0(control), 125, 250, 500 μl. MJ and SA respectively dissolved in an adequate volume of ethanol 96% and NaOH 0/1 N. The solutions were filtered through membrane filter (pore size: 0.2 μm) and stored below −20°C.
Treatments with MJ and SA
For experiments 250 mL Erlenmeyer flasks containing 50 mL liquid medium (1/2 MS) was inoculated with six cm$^2$ pieces of hairy roots. The cultures were shaken at 110 rpm and 25°C, with 16h photoperiod. Treatments with MJ (0, 50, 100, 200 μl) and SA (0, 125, 250, 500 μl) were done 28 days after been shaking and transfer, when hairy roots were in the active growth phase. The treated and non treated (control) hairy roots were harvested 48h after treatment with elicitors.

Noradrenalin extraction and assay
After being air-dried and crushed into powder, for extraction of Noradrenalin 100 mg of the accurately weighed hairy root sample was extracted with 5 ml of 0.1 M HCl solution in an ultrasonator for 1.5 h. The extract was then filtered through a filter paper and a 0.45 mm filter membrane to be ready for analysis (Chen et al. 2003). Total noradrenalin was determined by HPLC. The HPLC system used was a Caniver (Berlin, Germany) with C18 column (25cm × 4.6mm) and UV detector. The mobile phase consisted of 0.02 M KH2PO4 (%95) solution, acetonitrile (%5), PH3.0 and 280nm detection wavelength.

Statistical analysis
All analyses were done on a completely randomized design. All data obtained were determined by using analysis of variance with the Stat View Software package SAS 9.1 and the mean differences were compared by lowest standard deviations (L.S.D.) test. Each elicitors was examined in four levels and three repeats (n= 12) at the probability $P < 0.05$, considered significantly different.

Result and discussion

Induction of hairy roots
When the two-week old leaf explants were inoculated with freshly grown A. rhizogenes ATCC15834 suspensions in 1/2MS medium, hairy roots were induced directly from the cut edges of leaf (Fig.1). The first roots were visible 10 days after inoculation. Transformed root morphology of the primary cultures was variable, possibly due to slight differences in wound induced phytohormone production or differences in rolB expression (Palazón et al. 1998). Transformed hairy roots are stable and highly productive under hormone-free culture conditions. The fast growth, low doubling time, ease of maintenance and ability to synthesize a range of chemical compounds of hairy root cultures offer additional advantages as continuous sources for the production of valuable secondary metabolites (Tripathi 2003).

Fig1: induction of hairy roots in Portulaca oleracea by AR15834, a) produce of hairy roots in cutting place of leaf explants b) fast extension of transformed roots in solid 1/2 MS medium including sefotaksim antibiotic, c) transferred hairy roots to liquid 1/2 MS medium for improving growth

Sevon (2002) has summarized the most important alkaloids produced by hairy roots, including Atropa belladonna L., Catharanthus tricophyllus L., and Datura candida L. The presence of the rolB gene in the hairy root lines was tested by PCR amplification of the DNA using rolB forward and reverses primers. A. rhizogenes (colony PCR) served as the positive control and DNA from the non-transformed seedlings roots served as the negative control. All transformants showed presence of the 780bp rolB amplified product (Fig 2). Only the amplified products of the expected size (780bp) in the positive control and hairy root lines hybridized with the probe, confirming the identity of this amplification product and the transgenic were nature of the hairy root lines (Fig 2).
Fig 2: PCR-products of rolB gene in transformed root of Portulaca oleracea by Agrobacterim rhizogenes AR15834 in 0/9 agarose gel, well 1: molecular marker 1000 bp, wells 2-7: lines of transformed hairy roots, wells 8-9: lines of control root (nature roots), wells 10-11 positive control (plasmid DNA)

MJ

The results show that accumulation of noradrenalin was enhanced in the transformed roots, after the 48h treatment with different concentration of MJ, particularly with the highest and lowest concentrations of MJ, compared to no transformed roots (Table 1). Especially, MJ at a concentration of 200μm induced the accumulation of noradrenalin by approximately 8-fold as compared to the untreated control (Fig 3).

Table 1: Elicitation of noradrenalin productivity (mg/L) by purified MeJ

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>Mean of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenalin</td>
<td>0/013</td>
<td>0/0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>0/013</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0/0001</td>
</tr>
<tr>
<td>CV</td>
<td>-</td>
<td>4/38</td>
</tr>
</tbody>
</table>

**differences at \( P \leq 0.01 \) according to one-way analysis of variance (ANOVA).

Fig 3: Elicitation of noradrenaline productivity (mg/ L) by purified salicylic acid. Non similar words indicate significant differences at \( P \leq 0.01 \) according to one–way analysis of variance (ANOVA).

Mao et al. 2006 reported similar results that Me increased the levels of gossypol and its methylated derivatives and their studies have demonstrated that MeJ up regulates transcription of the deltacadinene synthase A gene that encodes the first enzyme in the gossypol biosynthetic pathway. Jasmonates (e.g. MJ) have been reported to be elicitor signal transducers for the production of plant secondary metabolites (Gundlach et al. 1992). They induce accumulation of compounds belonging to different structural classes, including phenolics, terpenoids, alkaloids and others. A jasmonate-responsive transcription factor linking
plant stress responses to changes in metabolism was isolated from Catharanthus roseus (van der Fits and Memelink, 2000). A DNA microarray analysis confirmed the transcriptional reprogramming of plant cells for multiple genes of secondary metabolite biosynthesis in response to MJ (Schenk et al. 2000; Suzuki et al. 2005). Malarz et al. 2006 and Zayed and Wink 2004, respectively, reported on MJ increased accumulation of the three monitored sesquiterpene lactones in the hairy roots of Cichorium intybus and hyoscyamine concentration in hairy root cultures of Brugmansia suaveolens by approximately 25-fold as compared to the untreated control.

SA
In this study results show that SA did not affect on accumulation of noradrenalin in transformed roots of Portulaca oleracea, 48h after addition of SA to the nutrient medium, rather than no transformed roots (Table 2). None of the concentration of SA did have significant difference with control (Fig 4).

Table 2: Elicitation of noradrenalin productivity (mg/ L) by purified salicylic acid

<table>
<thead>
<tr>
<th>Mean of squares</th>
<th>Source of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenalin</td>
<td>Treatment</td>
</tr>
<tr>
<td>0/003</td>
<td>3</td>
</tr>
<tr>
<td>0/001</td>
<td>8</td>
</tr>
<tr>
<td>26/2</td>
<td>CV</td>
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</tbody>
</table>

Non of treatment indicate significant differences at $P \leq 0.05$

Fig 4: Elicitation of noradrenalin productivity (mg/ L) by purified salicylic acid.

In cotton hairy root cultures, SA did not increase the production of gossypol or its two methylated forms, at least at the time points and the concentrations tested (Frankfater et al. 2009). Xu et al.(2004) reported similar results, although they used a spectrophotometric method that quantifies the total pool of terpene aldehyes.

References


