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Improvement of alizarin production by different biotic elicitors in *Rubiatinctorum* by elicitation-infiltration method

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ABSTRACT

Background & Aim: *Rubiatinctorum* is one of the most well-known medicinal plants whose alizarin and other anthraquinones which are outstanding color agents with some trace of pharmaceutical properties are isolated from it. The objective of this study was to optimize alizarin production in intact plant of *R.tinctorum* by induction with biotic elicitors.
Experimental: To increase the synthesis of alizarin, bacterial (*Staphylococcus aureus* and *Bacillus cereus*) and fungal (*Fusarium oxysporum* and *Aspergillus fumigatus*) elicitors were injected to the intact plants directly by fine needles, named elicitation-infiltration method. Then samples were analyzed through standard addition method by UV-visible spectroscopy.
Results: *Staphyloccus aureus* (1McFarland) was the most effective one on biomass accumulation. Furthermore, among fungal elicitors *Aspergillusfumigatus* (0.4 mg total sugar/mL) revealed the most significant help for biomass increase. Applying bacterial elicitors imposed a dramatic increase in alizarin yield in all concentrations. The most marked increase (5 fold) was for 0.5 McFarland of *Bacillus cereus*. In addition, *Fusarium oxysporum* indicated outstanding results for alizarin production's enhancement.
Recommended applications/industries: To the author's knowledge, the application of elicitation-infiltration method for increasing the alizarin production is studied for the first time and according to the reported results, it can be a useful method for more investigations about improvement of secondary metabolites production in other plants.

1. Introduction

Nowadays, there has been an increasing interest in alternative therapies and therapeutic use of natural products, especially those which are derived from plants. This desire in drugs with plant origins is hidden within several reasons, such as inefficiency of conventional medicine, unsuitable consumption of synthetic drugs and inaccessibility of conventional pharmacological

treatment for a large percentage of the world's population and less side effects for natural products (Rates, 2001).

The secondary metabolites play major roles to adapt the plants to their environments. They have been explained as being antibiotic, antifungal, antiviral, anti-germinate and toxic for other plants. Due to their vast biological activities, plant secondary metabolites have been applied in traditional medicine for thousands of years. Recently, they comprise invaluable substances such as pharmaceutics, cosmetics, fine chemicals and dyes (Bourgaud *et al*., 2001). Anthraquinones are a kind of secondary metabolites which encompass numerous compounds, differing in the nature and positions of substituents groups. They are well-known constituents of *Rubiaceae* family which are isolated mainly from the roots, leaves and fruits. Many anthraquinones represent potential therapeutic value, because antimicrobial, insecticidal, antitumor, anti-congestive, hypotensive and sedative properties have been assigned to these compounds (Oliveira *et al*., 2007). Furthermore, another significant feature of anthraquinones is coloring as a dye. Alizarin, a prominent member of anthraquinones, is the main dye component of *Rubiatinctorum* (Dereksen and VanBeek, 2002). The chemical synthesis of alizarin although presented magnificent properties such as low cost and toxicity but revealed some debates about its applications and quality too. Chemically speaking, the process that converts anthracene to synthetic alizarin, generates several side products, mostly molecules with similar structures to alizarin, requiring extra purification stages (Ball, 2007). In addition, some painters have claimed that synthetic alizarin does not give pleasing, saturated and fiery tone that natural one gives (Gettens and Stout, 2011). Another significant reason for taking natural alizarin into our priorities lies in the fact that, synthetic one represents some hazardous signs such as: LD_{50} 0.3 g/kg (Choudhury and Muthu, 2014). So various researches have been performed to increase the yield of this invaluable dye either in cell/tissue culture or in the whole plants of *R. tinctorum* (Dereksen and Van Beek, 2002). Elicitors are compounds which stimulate any types of physiological stress on the plant. They are grouped into two categories in terms of origin: exogenous (substances originated from pathogens) and endogenous (substances originated from plants). Elicitors can be applied to enhance plant's secondary metabolite by interfering in biosynthetic pathways of commercially valuable compounds. The secondary metabolites are released due to defense responses which are started and activated by elicitors (Patel and Krishnamurthy, 2013). For example, *Aspergillusniger* and *Fusarium oxysporum* had promoted the yield of thiophene in *Tagetespatula* and anthocyanin in *Daucuscarota*, respectively (Namedeo, 2007; Suvamalatha*et al*., 1994). In addition, *Bacillus cereus* and *Staphyloccus aureus* affected the production of scopolamine in *Scopoliaparviflora* markedly (Guillon *et al*., 2006). Injection-infiltration method has been introduced to investigate the pathogenicity of plant invading microorganisms. Cell suspension of bacteria is injected to the leaves of plants by fine needles. The method has this advantage over tissue culturesbecause it can be carried out in intact plants (Klement, 1963). In this study, the standard addition was exploited in order to measure amounts of alizarin in the samples by means of spectrophotometer as it is proposed one of the most popular strategies for measuring anthraquinones levels (Derksen and VanBeek, 2002). In standard addition, measured amounts of analyte are added to the samples. From the increase in signal, it can be concluded that how much analyte is in the original sample. This procedure requires a linear response to analyte. Standard addition is significantly suitable when the sample composition is

unknown or complex, affecting the analytical signal (Harris, 2010).

The purpose of the present study is probing the effect of fungal (*F. oxysporum* and *A. fumigatus*) and bacterial (*S. aureus* and *B. cereus*) elicitors on the biomass and alizarincontent in intact plants of *R.tinctorum*.

2. Materials and Methods

2.1. Seed sterilization and germination

The seeds of *R.tinctorum* were sterilized by soaking in NaClO 2% (v/v) for 10mins and 1 min, respectively. They were washed for 3 times with sterile distilled water. Sterile seeds were placed on MS medium for germination. The cultures were kept in the seed germinator, setting the following condition: 16h/8h light/dark cycle and temperature 25±2°C (Zanousi *et al*., 2012). The sample plants were 2-month old. It was tried to choose the plants with similar physiological and morphological features.

2.2. Preparation of elicitors

2.2.1. Preparation of fungal elicitors. Firstly, one individual colony of the desired fungi (*F. oxysporum* and *A. fumigatus*) was moved to a 250 ml Erlenemeyer flask which contained 50 ml of PDB (Potato Dextrose Broth) media by a sterile spatula. Flasks were kept on an incubator shaker, rotating at 200 rpm at 25±2 °C, 16h/8h and light/dark cycle for 6 days. The broths were past throw Whatman No.1 filter paper and the remaining were centrifuged at 5000 rpm for 15 min. The fungal elicitors were prepared throw a technique suggested by Farakya *et al* (Baldi and Dixit, 2007). To determine the concentration of fungal elicitors, phenol-sulfuric acid method was exploited to reach to two different concentrations (0.2 and 0.4 mg total sugar/ml) of fungal elicitors (Dubios *et al.*, 1956).

2.2.2. Preparation of bacterial elicitors. *B.cereus* was cultured in Nutrient Broth (NB) at 27°C and 120 rpm for 24 hours and also *S.aureus* was cultured in tryptic soy broth (TSB) applying the same condition (Shakeran *et al*., 2015). Like fungal elicitors, two different bacterial concentrations were applied to elicit: 0.5 and 1 McFarland. These concentrations were prepared by McFarland turbidity standard method (Sutton, 2011).

2.3. Elicitation procedure

As mentioned before, elicitation-infiltration procedure had been introduced as a rapid assay for detecting the pathogenicity of a microorganism. This procedure is performed as follows: older leaves of the 2-month old-plants were selected to inject the elicitors. The elicitors were injected to lateral veins of the leaves, where the tissues were thick enough for injection. 3 ml of each elicitors were injected to intracellular spaces of the *R. tinctorum* leaves by sterile fine needles. 3 mL of sterile distilled water were injected to leaves of the control samples. The impacts of each elicitor were assayed on biomass and alizarin yield at 48 h after elicitation (Klement, 1963). All tests were repeated in triplicate.

2.4. Measurement of fresh and dry weight of intact plants

The fresh weight of the samples was measured after 48 injecting the elicitors to the plants, comparing the results with the controls. Then samples were dried in the darkness at room temperature for 5 days before the measurement of dry weight of the samples.

2.5. Extraction and assessment of alizarin

The 1.0 g of dried samples were extracted and purified to assay for alizarin yield. The samples were ground in 2 ml toluene in mortar. The mortar was washed with 1 mL methanol which was transferred to

the test tubes. Then the extracts were subjected to centrifugation at 5900 rpm for 10 min. The supernatant was removed and kept. These steps were performed repeatedly. The supernatant of the samples were dried under nitrogen gas and stored at -20°C (Smith *et al*., 1997). As mentioned before, in the present study Standard addition was exploited to measure amounts of alizarin in the samples. Matrix effect is a common effect in this method, revealing a change in the analytical signal caused by anything in the sample other than analyte. The prolonged step by step procedure of this method was followed by Daniel Harris (2010).

The procedure for standard additions is to split the sample into several even aliquots inseparate volumetric flasks of the same volume. The first flask is then diluted to volume with the selected diluent. A standard containing the analyte is then added in increasing volumes to the subsequent flasks and each flask is then diluted to volume with the selected diluent. The instrument response is then measured for all of the diluted solutions and the data is plotted with volume standard added in the x-axis and instrument response in the y-axis. Linear regression is performed and the slope (m) and y-intercept (b) of the calibration curve are used to calculate the concentration of analyte in the sample. From the linear regression:

$$S = mVs + b \qquad \text{[Equation 1]}$$

Where S is instrument response (signal) and Vs is volume of standard. Conceptually, if the curve started where the instrument response is zero, the volume of standard [(Vs)0] from that point to the point of the first solution on the curve (x = 0) contains the same amount of analyte as the sample. So:

$$Vx \times Cx = [(Vs)0] \times C_S \qquad \text{[Equation 2]}$$

Where Vx is the volume of the sample aliquot

Cx = concentration of the sample

Cs = concentration of the standard

Combining Equation 1 and Equation 2 and solving for Cx results in: $Cx = \frac{bCs}{mVx}$

And one can then calculate the concentration of analyte in the sample from the slope and intercept of the standard addition calibration curve.

2.6. Data analysis

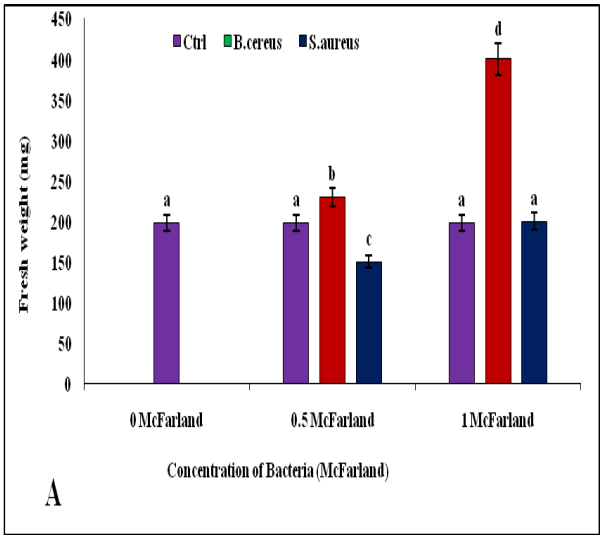
In this experiment, 324 plants of *R.tinctorum* were chosen for injection. Two different concentrations were selected for each bacterial (0.5 and 1 McFarland) and fungal (0.2 and 0.4mg total/mL) elicitors. The fresh weight, dry weight and alizarin content were measured in all of the samples. For each treatment, 3 plants of *R.tinctorum* were prepared for the injection. All experiments were performed in triplicate. The variance of the results was analyzed by Duncan test of SPSS software version 20 (SPSS Inc., Chicago, IL). In this study, univariate procedure at $P < 0.05$ was chosen (Shakeran *et al.*, 2015).

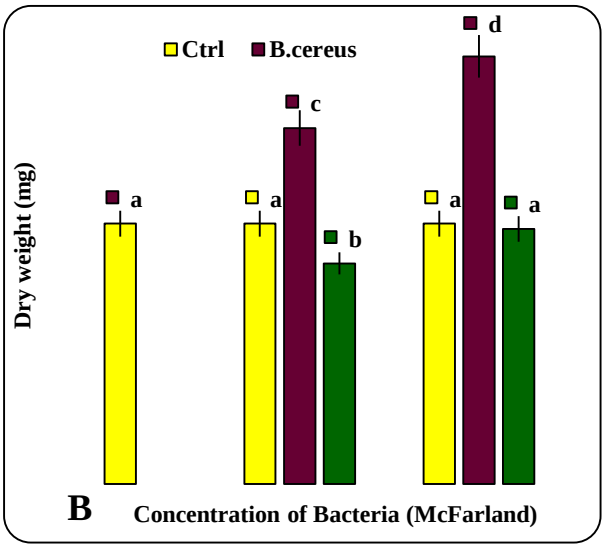
3. Results and discussion

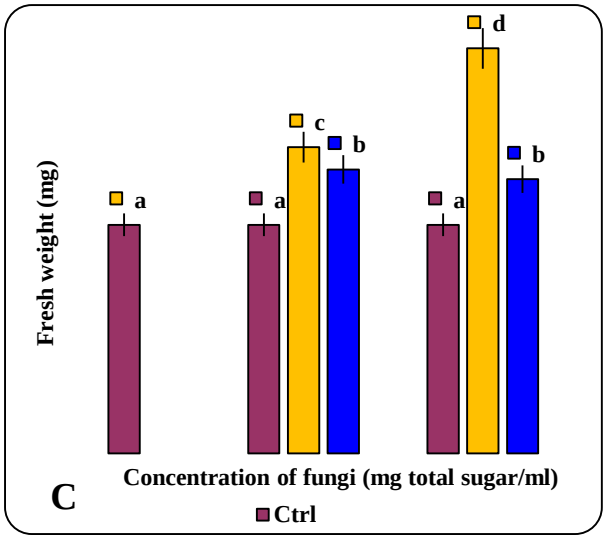
3.1. Effects of biotic elicitors on biomass accumulation

This study investigated the effect of elicitors on the biomass of intact plants. The results indicated that the highest yield of fresh and dry weight appeared after elicitation by 0.5 McFarland of *B.cereus* whereas *S.aureus* slightly decreased the biomass of the samples in comparison to controls (Figures1A and 1B). *B.cereus* is likely famous as a plant growth promoting rhizobacterium and can produce indole acetic acid which increases the growth rate of the roots, improving the water and inorganic substance uptake (Abdul Aziz *et al.*, 2012). The slight decrease in biomass accumulation by *B.cereus* is due to the destruction or lysis of the cells as

a consequence of an extreme attack against this bacterium (Shakeran *et al.*, 2015). Furthermore, by applying fungal elicitors (*F.oxysporum* and *A. fumigatus*) to the intact plants, these results were manifested: The most effective elicitor was *F.oxysporum* with 0.4 mg total sugar/mLfor increasing the fresh weight of the samples, while other elicitors revealed moderate signs of promotion in this field. The most significant elicitors were *A.fumigatus* with 0.2 and 0.4mg total sugar/mLfor dry weight of the samples. Other elicitors had acceptable impact on the sample's dry weight (Figures 1C and 1D).







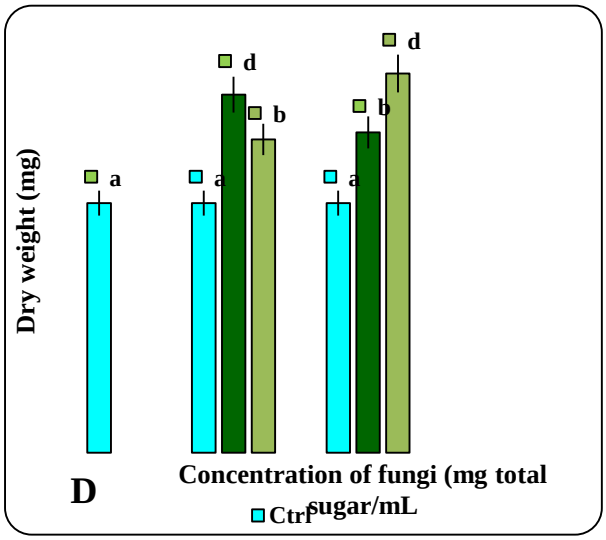


Fig 1. The effects of bacterial and fungal elicitors on fresh and dry weights of samples are presented by Fig 1A, Fig 1B, Fig 1C and Fig 1D, respectively. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

3.2. Effects of biotic elicitors on alizarin yield

As mentioned previously, standard addition method was exploited to quantify the alizarin level in the samples. Since there were numerous samples, one sample was chosen to depict its graph (Figure 2). As it is illustrated in the Figure 3A, 0.5 McFarland *B.cereus* imposes the greatest effect on alizarin production, in addition other bacterial elicitors revealed significant

impact on alizarin yield. The yield was equal for both *B.cereus* and *S.aureus* in 1 McFarland.

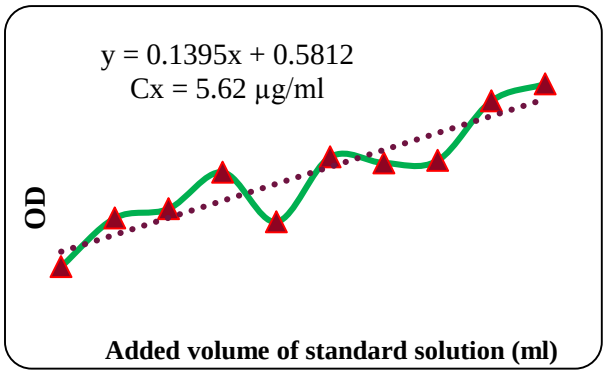


Fig 2. The present diagram illustrates the effect of 0.5 McFarland *B.cereus* on alizarin yield.

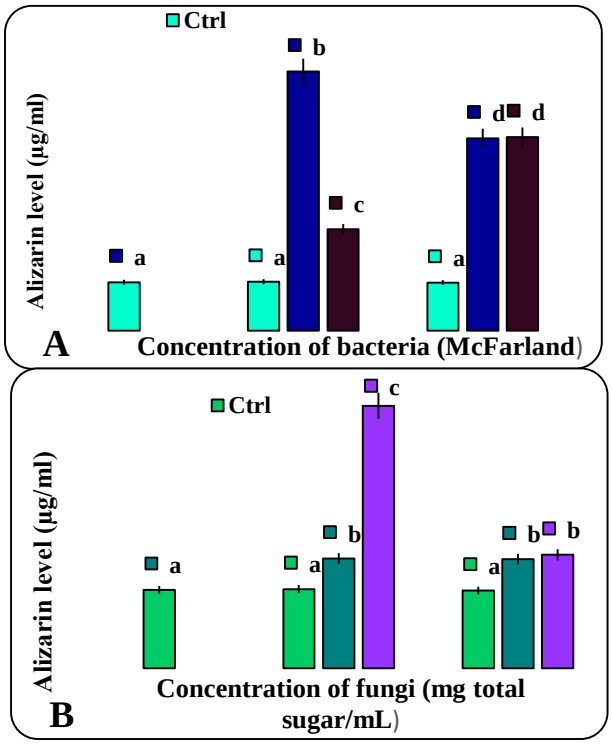


Fig 3. The effects of various biotic elicitors with different concentration on alizarin production of samples. Fig 3A and Fig 3B depict the effects of bacterial and fungal elicitors on alizarin level, respectively. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

Jung *et al.* (2003) revealed that scopolamine production was affected by adding *B.cereus* and *S.aureus* to hairy root cultures of *Scopoliaparviflora* after 12 hours, while neither of them did not show any marked impact on hyoscyamine yield within this period. Furthermore, *F.oxysporum*, *S.aureus*, *Aspergillus niger* and other elicitors were applied to hairy root cultures of *Tagetespatula*in order to enhance thiophene production. *F.oxysporum* drew the best results for improving alizarin yield that is in agreement with the findings of the present study (Figure 3B). The difference between efficiency of each elicitors lies in the fact that, these events are related to the unique signal transduction route for each elicitors in terms of their origins, concentration and even time periods (Buitelaar *et al*., 1992).

4. Conclusion

To summarize the results and draw a conclusion for the scope of the article we should mention, according to the findings, *B.cereus* is the most effective elicitor for increasing the alizarin yield and *F.oxysporum* stands in the second rank. For biomass accumulation, 0.4 mg total sugar/ml *F.oxysporum* had a great force to increase the fresh weight whereas 1 McFarland of *B.cereus* compels the samples to enhance the dry weight. Generally fungal elicitors possessed significant and promising results for increasing the alizarin yield in comparison to bacterial ones.

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