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Salicylic acid-induced changes to growth, flowering and flavonoids production in marigold plants

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Salicylic acid (SA) is a phenolic phytohormone that acts as a key regulator of the signaling network in plants under abiotic and biotic stresses. Also, SA exerts stimulatory effects on various physiological processes related to plant growth and development. The purpose of this study was to test the hypothesis that exogenous application of SA affects positively the growth and flowering of marigold plants (*Calendula officinalis* L.) and also induces an increase in total flavonoids content in the inflorescences of this medicinal species. Plants under greenhouse conditions were sprayed with SA (0.00; 0.25; 0.50 and 1.00 mM) for 3 consecutive days. The effects of SA in marigold were evaluated by the following parameters: leaf gas exchange, number of leaves per plant, leaf dry mass, leaf area, chlorophyll content, number of inflorescences per plant, fresh and dry mass of inflorescences and total flavonoid content in inflorescences. Application of SA in this concentration range resulted in linear increases on biomass accumulation, number of inflorescences and flavonoid content. Leaf gas exchange was not altered by SA application. These results showed that SA exogenous application before the reproductive stage resulted in higher biomass production of marigold plants and added significant value to the raw material by increasing total flavonoids content in the inflorescences.

Key words: *Calendula officinalis* L., growth regulator, medicinal plant, secondary metabolism.

INTRODUCTION

Calendula officinalis L. (Asteraceae), known as calendula or marigold, is an annual specie widely used around the world as a medicinal plant. The marigold inflorescences present essential oils, saponins, flavonoids and carotenoids, among other potentially active chemical constituents. However, flavonoids have a more important role in the pharmacological activity of inflorescences and are represented, in most cases, for the compounds quercetin and rutin, which are also markers to assess the quality of the raw material (Bilia et al., 2002; Rodrigues et al., 2004).

Salicylic acid (SA) is a phenolic compound of hormonal nature produced by plants and plays an important role in responses to several abiotic stresses and to pathogen attack (Noreen et al., 2009; He et al., 2002). SA has also been studied for its effects on various physiological processes related to growth and development of plants under normal conditions (no stress). Among these effects are the induction of flowering in herbaceous species (Hegazi and El-Shrayi, 2007), stimulation of root development, stomatal closure and reduced transpiration (Singh and Usha, 2003), reversal of the effects of abscisic acid (Davies, 2004), regulation of gravitropism (Hussein et al., 2007) and fruit ripening inhibition (Srivastava and

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Dwivedi, 2000).

The effect of SA as an endogenous regulator of flowering was demonstrated in a number of plant species belonging to different families (Hayath et al., 2007). In addition to regulate flowering time, SA also links defense responses and reproductive development (Martínez et al., 2004). Exogenous application of SA (1 and 2 mM) enhanced shoot, root and total plant dry weight under no salt stress in *C. officinalis,* besides providing an early flowering and high number of floral buds per plant (Bayat et al., 2012).

The molecular mechanisms involved in the flower-inducing activity of SA remain unclear. Phenolic substances could act as energetic regulators in oxidative phosphorylation or act together with growth substances, since the metabolism of indole-3-acetic acid (IAA) can be changed by the presence of phenolic substances (Grambow and Langenbeck-Schwich, 1983). Genetic studies pointed out to SA as a regulator of flowering time that interacts with both the photoperiod-dependent and autonomous pathways (Martinéz et al., 2004). In the short day specie, *Pharbitis nil*, the flowering is induced by poor nutrition stress. However, flowering under this condition was prevented by a phenylalanine-ammonia-lyase (PAL) inhibitor and restored by SA application. Such behavior was observed only under stress conditions; thus it appears that SA might be necessary but not sufficient to induce flowering (Wada et al., 2010).

Studies concerning the effect of plant growth regulators on the accumulation of secondary metabolites in medicinal plants have been carried out in order to increase the medicinal and commercial value of these species beyond the primary objective of evaluating the effects of regulators on plant growth and development. Specifically, regarding SA exogenous application, it may also induce the expression of many defense genes which encode particular enzymes of secondary metabolic pathway to form bioactive compounds such as phenolics (Ali et al., 2007). A significant increase in the synthesis of flavonoids in response to application of SA was observed in various medicinal plant species like *Matricaria chamomilla* (Kováčik et al., 2009), *Taraxacum officinale* (Kim et al., 2009), *Zingiber officina*le (Ghasemzadeh and Jaafar, 2012) and *Silybum marianum* (Khalili et al., 2009).

Kováčik et al. (2009) pointed out that the SA concentrations tested in *M. chamomilla* induced both growth-promoting (50 µM) and growth-inhibiting effects (250 mM). Also, the higher SA dose resulted in the rise of the activity of the enzyme phenylalanine-ammonia-lyase (PAL), followed by an increase in the accumulation of soluble phenolic compounds and lignin.

The aim of this study was to verify if the exogenous application of salicylic acid (SA) would affect positively the growth and flowering of marigold plants (*C. officinalis* L.) and increase the accumulation of total flavonoids in the inflorescences of this important medicinal plant species.

MATERIALS AND METHODS

Seedlings from marigold commercial seeds (*C. officinalis* L.) were grown in trays under greenhouse conditions without temperature control. After 30 to 35 days, the seedlings (15 cm in size) were planted in 5 L pots containing commercial substrate, soil and sand in a ratio of 1:1:1 (v/v/v). The pots were irrigated daily in order to maintain the substrate at field capacity throughout the experiment. The application of salicylic acid - SA (Sigma Aldrich, St Louis, MO, USA) was performed 60 days after sowing (before the reproductive stage) for 3 consecutive days (Martin-Méx et al., 2005). The different concentrations of SA (0.00, 0.25, 0.50 and 1.00 mM) were established according to Kováčik et al. (2009). SA treatments were carried out by spraying the aerial part of the plants with water-based solutions supplemented with Tween 20 (0.05 ml/liter of solution) until drip point.

The harvest of the inflorescences were initiated 90 days after sowing (DAS) with the emergence of the first flowers being held twice a week until plant senescence (120 DAS). At each harvest, the removed inflorescences were dried in an oven with air circulation at 40°C until achieving constant weight to determine the dry mass. At the end of the collection period, the values of all samples were summed to give the total dry mass of the inflorescences (g plant^{-1}). The effect of SA in plants was evaluated using the following variables: leaf gas exchange, number of leaves per plant, leaf dry mass, leaf area, chlorophyll content, number of inflorescences per plant, fresh and dry mass of the inflorescences and total flavonoid content in the inflorescences.

Leaf gas exchange was measured with a portable infrared gas analyser meter (model CIRAS-2 PPSystem) 48 h after application of SA. The measurements of CO_2 assimilation (A - micromol CO_2 m^{-2} s^{-1}), stomatal conductance (gs - mmol H_2O m^{-2} s^{-1}), transpiration (E - mmol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (Ci - micromol CO_2 L^{-1}) were made on visually healthy and fully expanded leaves of four plants in each treatment.

Leaf number per plant, leaf dry mass (g), leaf area (cm^2) and chlorophyll content were evaluated at the beginning of flowering stage, coinciding with the time of maximum biomass accumulation. The leaf dry mass (g) was measured after drying the samples at 60°C for 72 h. Leaf area was determined with a LI-3000A portable area meter (Li-Cor, Lincoln, NE, USA) in five plants per treatment. Chlorophyll content was determined with a manual meter (CCM-200 Opti-Sciences) in 3 leaves per plant, for each treatment.

Total flavonoid content was estimated by colorimetric aluminum chloride method according to Verlag (1978), using quercetin as the standard for the calibration curve. The total flavonoid concentration (µg mL^{-1}) was determined spectrophotometrically at 420 nm. The experiment was arranged in a completely randomized design with 4 treatments and 10 replicates (individual plants) per treatment. Data were tabulated and submitted to variance and regression analysis using the statistical program ASSISTAT (Silva, 2010) to evaluate the variables on different concentrations of SA.

RESULTS AND DISCUSSION

The exogenous application of SA resulted in significant effects on biomass accumulation of marigold plants as shown by the positive linear relationship between the leaf

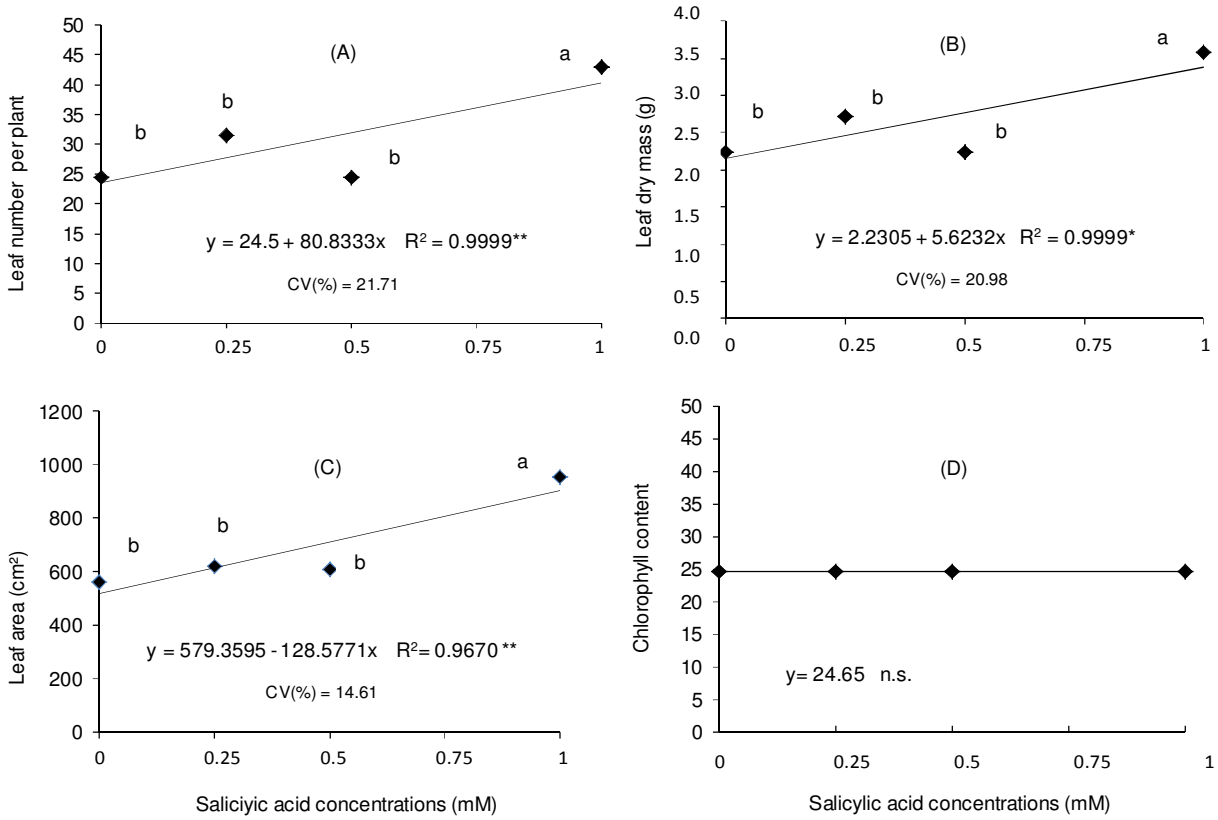

Figure 1. Leaf number per plant (A), leaf dry mass (B), leaf area (C) and chlorophyll content (D) in marigold plants treated with increasing doses of salicylic acid (0.00, 0.25, 0.50 and 1.00 mM).

variables and the level of SA applied on the aerial parts (Figure 1A to C). Plants treated with 1 mM of SA showed the higher leaf number, leaf dry mass and leaf area (79, 16%; 69, 24% and 59, 95% of increase, respectively) in relation to the control plants. These results corroborate the findings of Khan et al. (2003) who reported increases in leaf area and shoot dry weight of soybean and corn plants treated with 10^{-5} M of SA.

It is proposed that the increase in bioproductivity of plants is mainly due to the positive effect of SA on root length and its density (Larqué-Saavedra and Martin-Méx, 2007). There is evidence of a cross-talk between the SA and auxin signalling pathways during plant vegetative growth (Rivas-San and Plascencia, 2011). On the other hand, the promotion of wheat growth in response to the application of SA was attributed to the increase of photosynthetic tissues, since there was a positive correlation between photosynthesis and leaf area on wheat plants treated with SA, both under stress and normal growth conditions (Arfan et al., 2007).

The chlorophyll content observed in SA treated plants did not differ significantly from the control plants (Figure 1D). Increases or decreases in levels of photosynthetic pigments (chlorophylls and carotenoids) after SA applications were reported to be dependent upon the species and cultivar (Arfan et al., 2007). However, our results agree with the observations made by Khan et al. (2003), who also found no changes in chlorophyll content in leaves of soybean and corn treated with SA. The gas exchange characteristics of marigold plants were not altered by the application of SA (Figure 2). Although this compound was effective in increasing the photosynthesis rate in soybean and corn (Khan et al., 2003) and in tomato (Stevens et al., 2006), the same effect was not observed in our experiment, despite of high values for stomatal conductance (gs) and transpiration rate (E) at a low concentration of the SA (0.25 mM). It is suggested that the observed increase in photosynthesis rate in plants sprayed with SA can be assigned to metabolic changes at the chloroplasts level (efficiency of photosystem II, Rubisco enzyme activity and supply of ATP and NADPH for the carbon reduction cycle) (Rivas-San and Plasencia, 2011). However, the stimulatory effect of SA on gas exchange parameters and plant development is dependent on several factors such as application mode, exposure time and ontogenetic stage

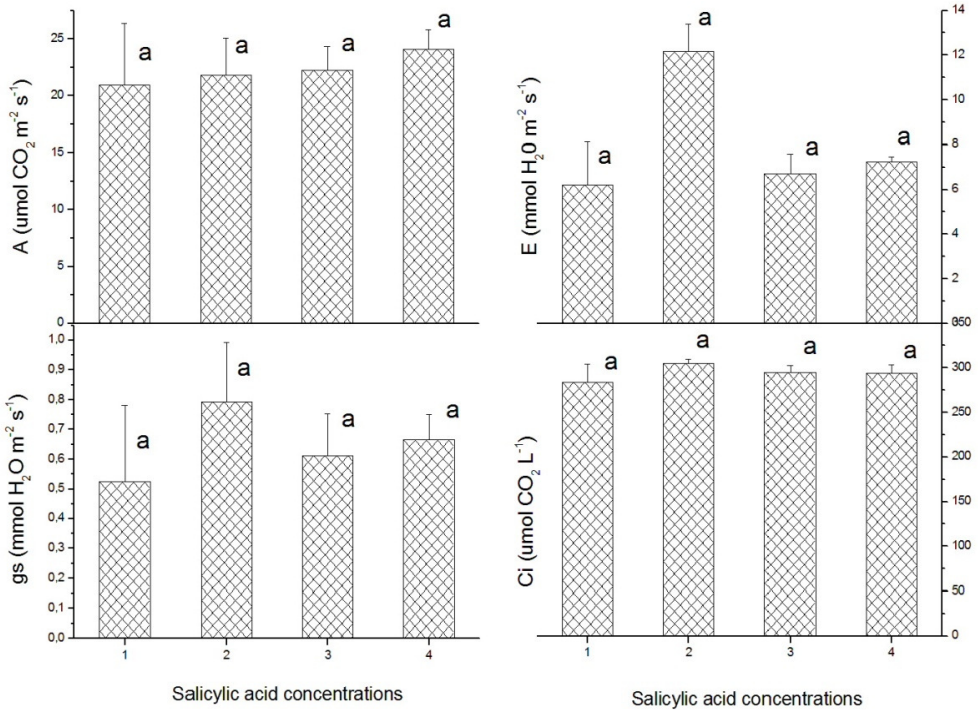

Figure 2. Net CO_2 assimilation (A), transpiration (E), stomatal conductance (gs) and internal CO_2 concentration (Ci) in marigold plants treated with increasing doses of salicylic acid (1= 0 mM ; 2= 0.25 mM ; 3= 0.5 mM and 4= 1.0 mM).

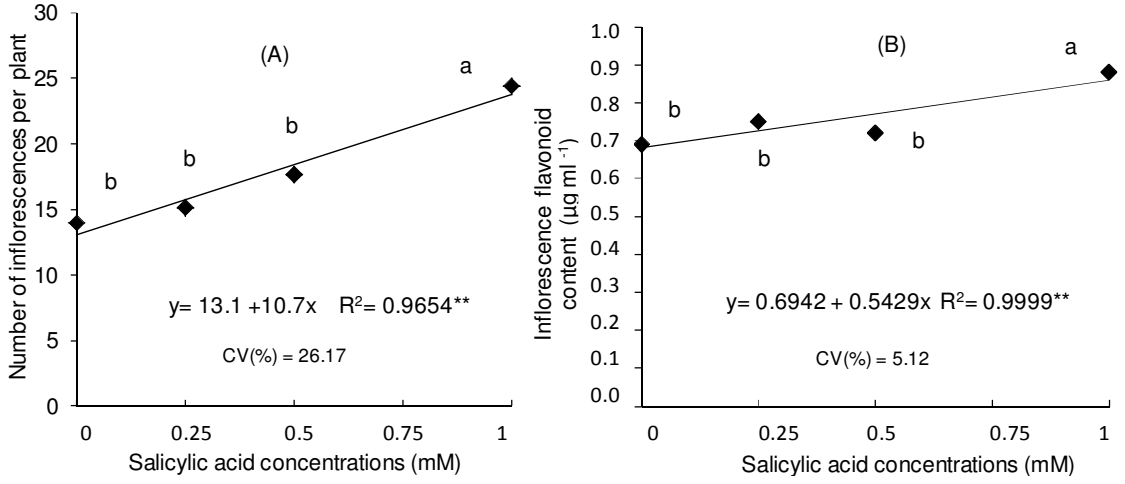

Figure 3. Number of inflorescences (A) and total flavonoid content in inflorescences (B) of marigold plants treated with increasing concentrations of salicylic acid (0.00, 0.25, 0.50 and 1.00 mM).

of the plant (Vanacker et al., 2001; Horváth et al., 2007). Besides, the effective concentrations of SA differ among species and their domestication stage (Arfan et al., 2007).

The number of inflorescences of the marigold plants was significantly higher at 1 mM of SA (Figure 3A). This result is in agreement with those of Bayat et al. (2012) who studied the effect of exogenous salicylic acid on growth of *C. officinalis* under salinity stress. Arfan et al. (2007) reported that the more effective SA concentrations

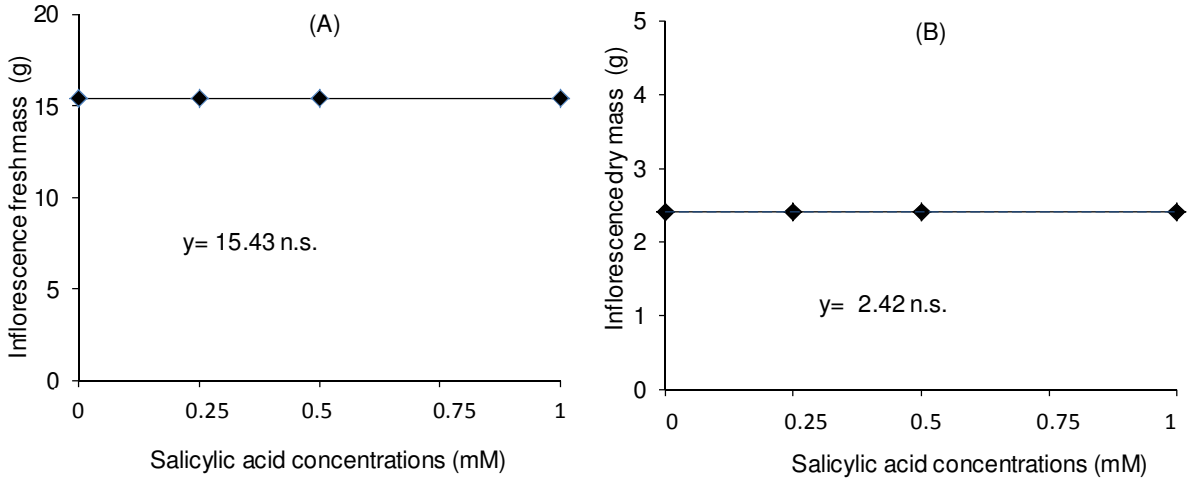

Figure 4. Inflorescence fresh (A) and dry mass (B) of marigold plants treated with increasing doses of salicylic acid (0.00, 0.25, 0.50 and 1.00 mM).

for promoting grain yield in wheat were 0.75 and 0.25 mM under normal and salinity conditions, respectively. The flowering promoting effect after SA application can also be indirect as SA alters the synthesis and/or signaling pathways of other plant hormones including jasmonic acid, ethylene and auxin (Vlot et al., 2009). In addition, the exogenous application of SA can rise the content of endogenous bioactive GA in response, changing the hormonal status of the plant (Mukherjee and Kumar, 2007; Kim et al., 2009). Increased content of endogenous SA as a result of its exogenous application were correlated to the positive influence on the plant growth and flowering (Kim et al., 2009). Although the SA doses within the range used in this study provided a greater number of inflorescences per plant (Figure 3A), there were no changes in fresh and dry mass of the inflorescences (Figure 4A and B).

We have observed a significant higher total flavonoid content in the marigold inflorescences at 1 mM SA (Figure 3B). Flavonoid levels in plants are significantly affected by plant growth regulators (PGRs) (Klessig and Malamy, 1994). According to Kim et al. (2009), the total flavonoid content in plants of *Taraxacum officinale* also increased significantly in response to the application of plant growth regulators such as SA, cytokinin and giberelic acid (GA_3), which demonstrates the effect of these substances on the biosynthesis of secondary metabolites.

Increased endogenous levels of SA can trigger cell-signaling pathways which regulate the expression of genes encoding enzymes related to the phenylpropanoid pathway production (among them the flavonoids), increasing the amount or the activity of these enzymes. For example, the chalcone synthase activity (the first enzyme to branch off from phenylpropanoid metabolism to flavonoid metabolism) was increased in plants treated with SA (Ghasemzadeh et al., 2012). Based on the results presented here, it can be concluded that the use of SA constitutes a valuable crop management technique to enhance the number of inflorescences in marigold plants. In addition to the positive effect on inflorescence production, the exogenous application of SA increased content of flavonoids in the inflorescences, which may result in higher economic value of the raw material used for manufacturing phytochemicals.

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