Article

Effect of culture conditions on the production of phenols, total flavonoids and their antioxidant capacity in arnica (*Heterotheca inuloides*)

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Abstract

At present, *Heterotheca inuloides* or arnica for its common name, has been used for its anti-inflammatory activity, in addition, this plant has other applications as a treatment against rheumatism, gastrointestinal disorders, among others. These therapeutic properties are due to secondary metabolites such as phenols and flavonoids. The concentration of these compounds can be influenced by biotic or abiotic stress, where the culture conditions such as relative humidity, the temperature of the environment and even the type of irrigation, represent an abiotic stress. That is why the objective of this work is to compare different culture conditions on the production of phenols, total flavonoids and their antioxidant capacity in arnica (*Heterotheca inuloides*). To achieve this goal, arnica plants were commercially obtained, placing 14 plants in a greenhouse and 14 plants in open-pit culture. In addition, each of these farming systems had two types of irrigation systems, the first with water with nutrient solution and the second with aquaculture wastewater. Also, it was elicited with salicylic acid at the concentrations 0.0 mM, 0.5 mM and 1 mM, twice every 14 days. The concentration of phenols, total flavonoids and antioxidant capacity was determined before each elicitation. Maximum phenolic compounds were present in plants grown in the field, irrigated with nutrient solution and elicited at 0.5 mM. The total flavonoids and antioxidant activity were present in greenhouse plants, irrigated with nutrient solution, without effect on elicitation. According to some of the results obtained I know that it concludes that the cultivation conditions for the production of phenols are not different for the production of flavonoids and the antioxidant capacity of said compounds.

Keywords: antioxidant capacity, culture conditions, phenols, flavonoids.

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Introduction

The cultivation of medicinal plants is increasing due to the increase in their use for therapeutic purposes and in the production of formulas for personal care and health products based on them. The 40% of the Mexican population uses medicinal plants as the only alternative for the treatment of diseases, being the main therapeutic resource in Mexico (Fernández *et al*., 2008). Today there are production manuals of medicinal plants where the requirements for cultivation are specified (CONAFOR, 2009; Villavicencio *et al*., 2010) however, there is no monitoring of the concentrations or conditions of the active principle.

The arnica (*Heterotheca inuloides*) is a plant commonly used as an anti-inflammatory. However, it is also used for the treatment of rheumatism, gastrointestinal problems and has now been used as an alternative treatment against cancer (Alonso-Castro *et al*., 2011) and diabetes (Andrade-Cetto and Heinrich, 2005; Johnson *et al*., 2006). The compounds that confer these medicinal properties are mainly sesquiterpenes, phytosterols and flavonides (Delgado *et al*., 2001). This plant is found in the cold and temperate regions of Mexico (Guerrero-Hernández *et al*., 2014; Gutiérrez and Solano, 2014). In Mexico, it is considered one of the most demanded medicinal plants (García de Alba *et al*., 2012; Juárez-Rosete *et al*., 2013; Monroy-Ortiz *et al*., 2013), it is obtained mainly wild, although small- scale cultivation has already started (Cesín-Vargas *et al*., 2010; Cristians *et al*., 2015).

The active compounds are those that confer the therapeutic and aromatic properties to said plants. These active compounds are made up of secondary metabolites, mainly phenolic compounds that work in the plant against oxidative damage and flavonoids that provide protection against pathogens (Ávalos and Pérez-Urria, 2009). According to Petinatti (2012), the concentration of phenolic compounds is modified by biotic and abiotic stress to which the plant is subjected. Factors such as temperature, radiation, nutrition and irrigation are factors of abiotic stress that affect the concentration of phenols. Such is the case of Ruta graveolens plants, where phenols are affected by UV radiation Vialart *et al*. (2012).

In addition, water stress, considered as abiotic stress, is studied on its influence on the production of phenols and flavonoids. Some of the plants studied on this type of stress are plantain (*Musa* spp.) (Moreno- Bermúdez *et al*., 2017), rice (*Oryza sativa* L.) (Ramírez, 2017), among others. On the other hand, the influence of abiotic stress on aromatic plants can influence specific compounds, such as the effect of high radiation on the biosynthesis of carvacrol in oregano where it was possible to increase the concentration of this compound Teraza *et al*. (2014). According to this the objective of this work is to compare the culture conditions on the production of phenols, total flavonoids and their antioxidant capacity in arnica (*Heterotheca inuloides*).

Materials and methods

For the experimentation of this work, 28 plants of arnica (*Heterotheca inuloides*) were commercially obtained from a greenhouse near the Amazcala campus and were identified by the QMEX herbarium. They were transplanted in bags with inert substrate and left in acclimation for 5 days.

The experiment was carried out in the Amazcala campus of the Autonomous University of Queretaro during a period of 28 days. The experimental design was 2 x 2 x 3 factorial; that is, two types of cultivation, two types of irrigation and three concentrations of elicitor. The 14 plants were grown in Gothic greenhouses with overhead ventilation and 14 plants in the field, applying two types of irrigation in each crop; the first consisted of water with nutrient solution (fertilizer) and the second with aquaculture wastewater. In addition, two elicitations were carried out every 14 days with salicylic acid, at concentrations 0.5 mM, 1 mM and a control, with sampling before each application. The ambient temperature (°C) and the (%) of relative humidity were monitored during the entire period of experimentation.

Water quality

The water quality was determined by spectrophotometric methods. The analysis of nitrites (NO_2^--N) was by the diazotization method (HACH method 8507, 2010), nitrates (NO_3^--N) by the method of cadmium reduction (HACH method 8171, 2010) and total phosphorus (FT) by the molybdovanadate method (Method HACH 8048, 2010).

Extraction of phenolic compounds

The extraction of total phenolic compounds was carried out by the method of Hassan *et al*. (2011). 0.1 g of sample was weighed and extracted first in an aqueous solution with 50% methanol. Then a second extraction was carried out with 70% acetone. This method allows the extraction of extractable compounds in methanol and acetone.

Total phenolic compounds

The total phenols were determined spectrophotometrically by the Folin-Ciocalteu method described by Singlenton and Rossi (1965). This method is produced by the oxidation of the hydroxyl groups by the Folin reagent. This reagent is composed of a mixture of sodium tungstate and sodium molybdate in phosphoric acid. In the determination of total phenols, the 20% anhydrous sodium carbonate solution was made and the Folin-Ciocalteu reagent was prepared at 1 N. For the concentration curve the gallic acid was prepared at a final concentration of 0.1 mg/ml. The produced redox reaction generates a blue coloration detected at a wavelength of 765 nm.

Total flavonoids

For the quantification of flavonoids, a solution of 2-aminoethyldiphenyl borate was prepared and 50 µl of the extraction of phenols, the fraction of extractable phenols, was taken and 180 µl of methanol plus 20 µl of the solution prepared with 2-aminoethylphenyl borate were added. Sodium nitrite (NaNO2) at 5%, aluminum chloride (AlCl3) at 10%, sodium hydroxide (NaOH) at 1 M and the standard solution of catechin with methanol were prepared. The reading was made in a spectrophotometer at 404 nm and the flavonoid concentrations were obtained with a standard curve of catechin.

Antioxidant capacity, DPPH

The antioxidant capacity by the DPPH method is a widely used method based on the donation of a hydrogen atom or the formation of complexes (DPPH-H and DPPH-R) through the stability of the 1, 1-diphenyl-2-picrilhydraz radical. The concentrations of the antioxidant capacities for each plant were determined by the percentage of inhibition (IC50).

The determination of the antioxidant capacity by the DPPH method was prepared in DPPH reagent (1,1-diphenyl-2-picrilhydrzil) with methanol. It was prepared in DPPH reagent with methanol. Aliquots of 1 865 ml of the reagent were placed in 2 ml microtubes and 0.135 ml of the methanolic extract of each sample. It was allowed to stand for 30 min protected from light and the reading was performed at a wavelength of 480 nm.

Antioxidant capacity, FRAP

To determine the antioxidant capacity by the FRAP method, the reagent was prepared with the mixture of a 20 mM solution of iron trichloride (FeCl3), acetates buffer with anhydrous sodium acetate and sodium acetate trihydrate at a pH of 3.7 and finally TPTZ (tripyridyl-2-thiazide) was prepared at 10 mM dissolved in 40 mM hydrochloric acid, 1 865 ml of the FRAP reagent and 0.135 ml of the methanolic extract of the samples were placed in 2 ml microtubes. It was left to react for 30 min under the protection of light. The trolox was used for the calibration curve. The absorbance reading was performed at a wavelength of 630 nm.

Results and discussion

Determination of total phenols

The results of the concentration of total phenols are shown in Figure 3. The maximum concentration of phenolic compounds occurred in plants grown in the field, irrigated with water with nutrient solution and elicited at a concentration of 0.5 mM. However, there is no significant difference between each concentration of elicitation. On the other hand, a significant increase in concentration can be noted when plants are elicited for the first time. Also, the effect of the crop is evident due to the fact that the plants cultivated in the field presented a higher concentration, unlike the plants grown in the greenhouse. These results agree with Petinatti *et al*. (2012), where abiotic stress effectively modifies the concentration of secondary metabolites, in this case of phenolic compounds.

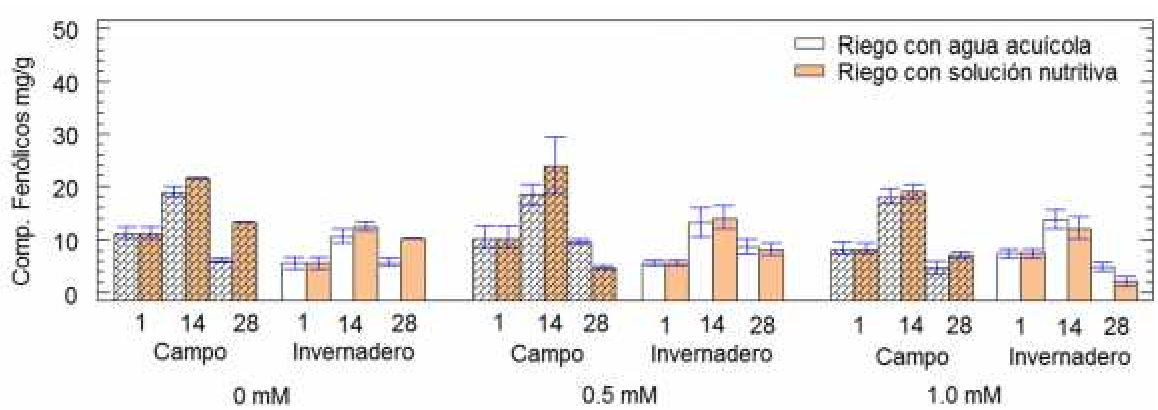

Figure 1. Concentration of total phenolic compounds (mg eq. ac. Gallic/g) in arnica cultivated in different conditions. The results are expressed in standard error. 1, 14, 28, days of sampling and elicitation.

The effect of field cultivation on the concentration of phenolic compounds can be influenced by the different temperatures observed in the monitoring of ambient temperature, observing maximum temperatures of 45 °C and minimum temperatures of 1.6 °C. In addition, the environmental temperature in the greenhouse does not present significant changes, maintaining its temperature range between 17 °C and 22 °C. In addition, the same behavior was observed in the relative humidity percentage. These results are shown in Figure 2.

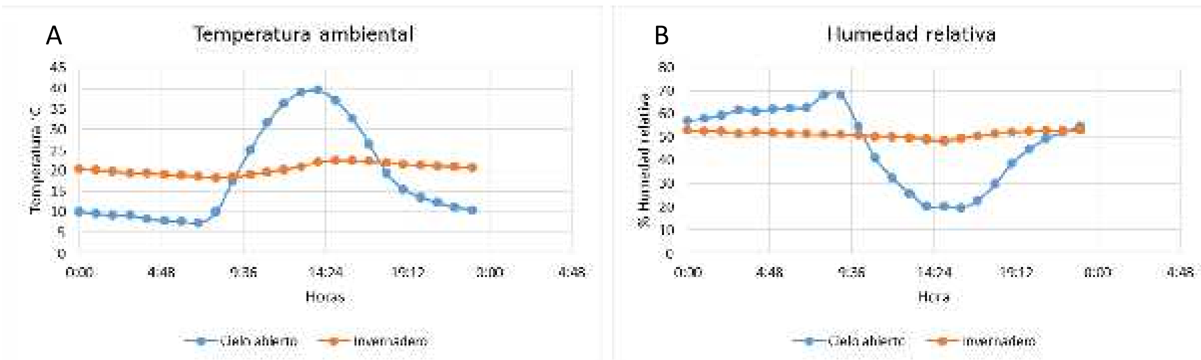

Figure 2. A. Environmental temperature; and B. Percentage of relative humidity in the two types of crops (greenhouse and field) in a 24-h cycle.

On the other hand, the increase in the concentration of total phenols in plants irrigated with nutrient solution is related to the concentration of nutrients supplied. The analysis of water quality showed that the concentration of nitrogen in water with nutrients is higher because it was prepared according to the needs of the plant. These results are presented in Table 1. In addition, the concentration of phosphates in the nutrient solution was higher, as expected. The influence of nutrition is studied in different plants such as oregano (*Lippia origanoides*) where the type of nutrition increases the yield of the essential oil Teles *et al*. (2014).

Table 1. Water quality of each irrigation system.

Type of irrigation	Nitrates mg/L	Nitrites mg/L	Phosphates mg/L
Nutritious solution	215.175	1.341	69.45
Aquaculture wastewater	118.375	0.321	14.35

Determination of total flavonoids

The total flavonoid concentrations in the arnica plants are presented in Figure 4. The results show a significant increase in the concentration of total flavonoids when they are grown under different conditions. The maximum concentrations are observed in plants grown in the greenhouse and irrigated with nutrient solution. No significant changes were observed between the concentrations elicited in each crop. However, the greatest increase in concentration is when plants are elicited for the first time.

On the other hand, it can be observed that only in the plants elicited at a concentration of 1 mM did they behave differently from the previous ones, that is, there is an increase in the concentration of flavonoids when the plants are grown in a greenhouse, irrigated with a solution nutritious and elicited twice with salicylic acid at a concentration of 1 mM. The effect of elicitation with salicylic acid was as expected since in different plants such as corn, an increase in the total biomass was observed, as well as the content of N, P, K, the content of phenols, flavonoids and punctual compounds such as the capasaicin Tucuch-Haas *et al*. (2017).

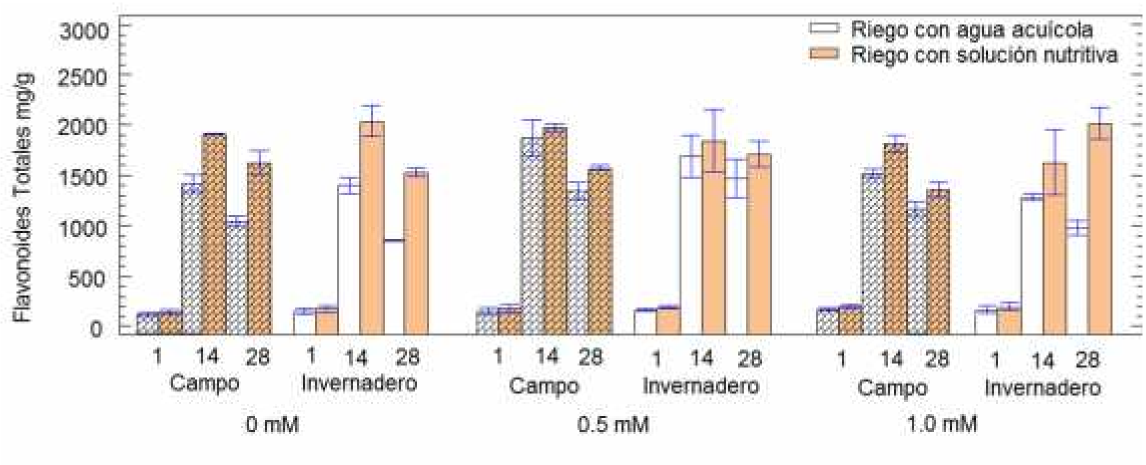

Figure 4. Concentration of total flavonoids (mg eq. Catechin/g) in arnica grown under different culture conditions. The results are expressed in standard error. 1, 14, 28, days of sampling and elicitation.

Antioxidant capacity, DPPH

The results of the antioxidant capacity are presented in Figure 5. The antioxidant capacities in the arnica plants were increasing in particular crops; that is, for plants grown in the field, irrigated with water with a nutrient solution and elicited at 0.0 mM on two occasions, they had an increase in antioxidant capacity. This result can be attributed to the variations in temperature and relative humidity to which they were subjected in field cultivation. For cultivated plants elicited at a concentration of 0.5 mM on two occasions, the increase in antioxidant capacity occurred in plants grown in the field, without significant differences between the type of crop. These results can be attributed to the response of the plant to the time and concentration of elicitation.

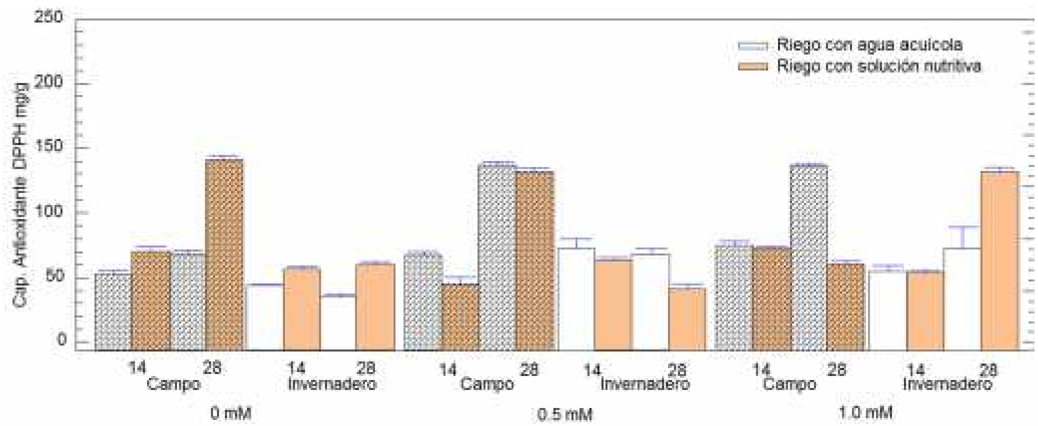

Figure 5. Antioxidant capacity (mg eq. Trolox/g) by the DPPH method in arnica plants grown in different conditions. The results are expressed in standard error. 14 and 28, days of sampling and elicitation.

The results of the antioxidant capacity in plants elicited at a concentration of 1 mM on two occasions, showed a significant increase when the plants are cultivated in the field and irrigated with aquaculture wastewater and in the plants grown in the greenhouse, irrigated with a nutritive solution. According to Rodriguez *et al*. (2017), *Heterotheca inuloides* contains a large variety of sesquiterpenes, flavonoids and terpenes, compounds that are present in the plant according to age, flowering time and even according to the geographical region of origin. For example, cadaleno and 4-methoxy-isocadalen are compounds that are absent in young branches of the plant. According to this, we attributed the results of the antioxidant activity by the DPPH method to the variety of compounds that could have an antioxidant activity in the different culture conditions.

Antioxidant capacity, FRAP

The results of the antioxidant capacity by the FRAP method are presented in Figure 6. The antioxidant capacity determined by the FRAP method showed significant differences between the type of crop, with field cultivation having the highest antioxidant capacity. However, the antioxidant capacity among irrigation systems was higher when the plant was irrigated with nutrient solution, but without effect by elicitation (0.0 mM). On the other hand, the antioxidant capacity in elicited plants at a concentration of 0.5 mM on two occasions, were greater when there is an irrigation system with aquaculture wastewater. In the same way, the antioxidant capacity of elicited plants at 1 mM was performed twice. These results show that there is a synergy between two types of abiotic stress, nutrition and elicitation.

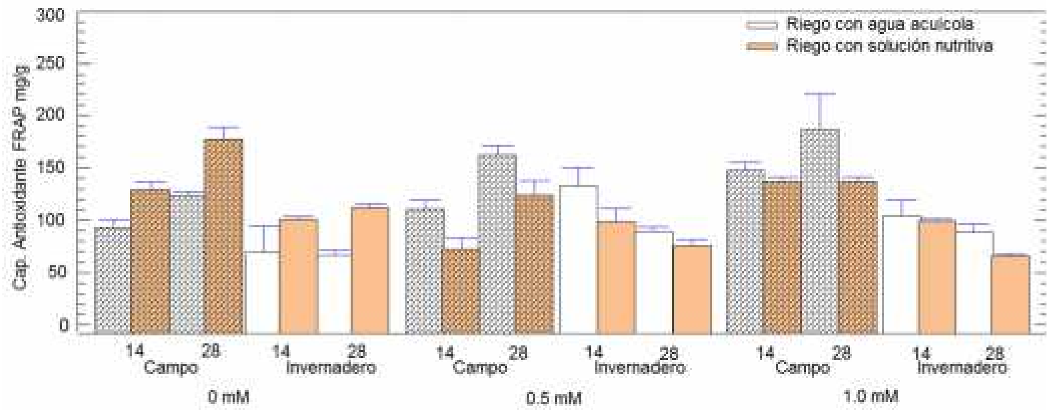

Figure 6. Antioxidant capacity (mg eq. Trolox/g) by the FRAP method in arnica plants grown in different conditions. The results are expressed in standard error. 14 and 28, days of sampling and elicitation.

According to these results, the antioxidant capacity is related to the abiotic stress to which the arnica plants were subjected. According to Hossain *et al*. (2010), the method of drying the plant material, in this case in *Lamiaceae* plants, has an influence on the antioxidant capacity measured by the FRAP method.

Conclusions

Field cultivation and concentration of the applied elicitor increases the concentration of phenolic compounds. The concentration of flavonoids increased significantly when the plant is cultivated in both types of crops (greenhouse and field), having differences between the type of irrigation. The antioxidant capacity is very different to the type of crop in which the plant is held due to the specific compounds and the type of antioxidant activity that they present.

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