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Variation of biochemical and antioxidant activity with respect to the phenological stage of *Tithonia diversifolia* Hemsl. (Asteraceae) populations

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ABSTRACT

Tithonia diversifolia is a medicinal plant traditionally used in the treatment of diabetes, diarrhea, menstrual cramps, malaria, hematomas, hepatitis, hepatomas and wounds. This work investigated the influence of environmental factors, phenological stage and genetic variability on the secondary metabolite profile and its antioxidant actions. Leaves were collected at the vegetative stage and in the reproductive stage, in five regions of southeastern Brazil. Phytochemical analyses, antioxidant activity and genetic markers as RAPD (Random Amplified Polymorphic DNA) were used to evaluate the variability between populations and to generate clusters. The highest contents of phenols, flavonoids and tannins were observed in ST (Santa Teresa) samples, as well as displayed great antioxidant activity. ST showed low genetic similarity to other populations (0.12), indicating high levels of genetic diversity and that clustering it is not positively correlated with the proximity of geographical locations. This analysis indicates that phytochemical and antioxidant variations between populations are influenced by genetic factors as well as environmental conditions.

1. Introduction

Tithonia diversifolia (Hemsl.) A. Gray (Asteraceae), a perennial shrub, is native to Mexico and grows in parts of Africa, Australia, Asia and some parts of America. Its extracts are traditionally used in the treatment of diabetes, diarrhea, menstrual cramps, malaria, hematomas, hepatitis, hepatomas and wound healing (Botsaris, 2007; Tona et al., 2000). These effects may be ascribed to the terpenoids and flavonoids presented in aerial parts of *T. diversifolia* (Chagas-Paula et al., 2012; Lee et al., 2011). Several studies investigated the anti-inflammatory, analgesic, antimalarial, antimicrobial and anti-diabetic activities and these investigations revealed the potential of its constituents in different pharmacological/therapeutic treatments (Chagas-Paula et al., 2012). Antioxidant properties of natural products have been investigated, such as the prevention of free radical generation, neutralizing radicals by non-enzymatic mechanisms, or enhancing the activity of endogenous antioxidants system (Zhu et al., 2004).

More than 150 compounds have been isolated from *T. diversifolia*, including sesquiterpenoids, diterpenoids and flavonoids (Chagas-Paula et al., 2012), thus its antioxidant activity is mainly attributed to phenolic compounds (Di Giacomo et al., 2015). Flavonoids are one of the main classes of *T. diversifolia* constituents that belong to the phenolic compounds group and exhibit anti-carcinogenic, anti-mutagenic, antioxidant and antimicrobial activity (Valdés et al., 2015; Valdez-Morales et al., 2014). The use of plants that are rich in phenolic compounds can prevent or delay the development of non-communicable diseases, such as cardiovascular diseases, diabetes and cancer, through processes involving reactive oxygen species (Ozcan et al., 2014). In addition, metabolites of dietary phenols exert modulatory effects in cells, including signaling cascades that are vital for cellular functions, such as growth, proliferation and apoptosis (Crozier et al., 2009).

Various works have demonstrated that the chemical constituent composition of plants, their content and their biological activities are dependent on environmental factors, such as temperature, precipitation, altitude, soil, season (Chirinos et al., 2013; Djerrad et al., 2015; Tlili et al., 2014); phenological stage (Pirbalouti et al., 2013) and genetics (Šamec et al., 2015). Biochemical and molecular markers, when used together, are often useful for establishing relationships between plant populations (Singh et al., 2012). There are no reports relating *T. diversifolia* genetic characterization with its environmental growth conditions and phenological stage, and the influence of these factors on the production of secondary metabolites. The present work aimed to

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characterize the chemical and genetic diversity of five *T. diversifolia* populations growing in the Southeast region of Brazil in the vegetative and reproductive stages, through preliminary phytochemical prospecting, and by assessing phenolic compound quantification, *in vitro* antioxidant activity and molecular markers.

2. Material and methods

2.1. Chemicals and reagents

ABTS, potassium persulfate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, linoleic acid, Tween 40, Folin–Ciocalteu reagent, Folin-Denis reagent, ferrozine, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) reagent, iron(III) chloride, iron (II) chloride, isoamyl alcohol, standards of gallic acid, tannic acid, rutin, Trolox, α-tocopherol, ferrous sulphate, ascorbic acid, β-mercaptoethanol and CTAB (Cetyl Trimethylammonium Bromide) were purchased from Sigma–Aldrich (Steinheim, Germany). Chloroform, ethanol (70%), methanol, isopropanol, glacial acetic acid, sodium carbonate, aluminum chloride were obtained from Vetec (Rio de Janeiro, Brazil). Proteinase-K and RNAse A were purchased from Ludwig Biotecnologia (Rio Grande do Sul, Brazil). GoTaq G2 Flexi amplification kits were obtained from Promega Brazil (São Paulo, Brazil). Primers were purchased from Molecular Brasil (Belo Horizonte, Brazil).

2.2. Plant material

The aerial parts of 10 *Tithonia diversifolia* adult specimens were randomly selected and collected at five localities of Southeastern Brazil: Colatina (COL) (19° 30′ 08.2" S; 40° 36′ 40.7" W), Muniz Freire (MF) (20° 28′ 09.9" S; 41° 24′ 14.7" W), Santa Teresa (ST) (19° 95′ 389"; 40° 55′ 783"W), Viana (VIA) (20° 25′ 23.184" S; 40° 28′ 37.2828" W) and Vitória (VIT) (20° 16′ 30.4716" S; 40° 18′ 17.5284" W), in September 2014 and April 2015. A voucher specimen (VIES 35297) was deposited in the Central Herbarium of Universidade Federal do Espírito Santo/VIES, Brazil. The samples were air-dried at room temperature for 5 days, pulverized and subsequently submitted to maceration in ethanol 70%.

2.3. Edaphoclimatic data

In order to correlate the chemical variability between localities with the geographic variation and environmental conditions, the data of altitude, accumulated precipitation and average temperature referring to the month of collection are described in Table 1 (Weather Information System/Incaper). The soil chemical analysis data are presented in Table 2, according to the methodology proposed by Silva (2009). The soil collection occurred simultaneously to the collection of the plant material.

Table 1
Environmental conditions data.

Location	Altitude (m)	Accumulated precipitation (mm)		Average temperature (°C)	
		2014	2015	2014	2015
ST	612	112.8	64.6	16.9	18.6
VIA	40	21.8	17.8	23.3	25.0
MF	603	17.9	76.4	21.8	23.0
VIT	10	83.2	55.6	23.3	25.1
COL	50	59.6	21.0	23.7	24.9

ST: Santa Teresa; MF: Muniz Freire; VIA: Viana; VIT: Vitória; COL: Colatina.

2.4. Genetic analysis using RAPD markers

Extraction of genomic DNA was based on the protocol described by Doyle (1987) with modifications. For this analysis, 100 mg of homogenized sample of 10 plants from each locality, previously frozen, were ground in liquid nitrogen with polyvinylpolypyrrolidone. The obtained powder was mixed with 1 mL of CTAB (Cetyl Trimethylammonium Bromide) extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8.00 and 20 mM EDTA pH 8.00), β-mercaptoethanol and proteinase-K, incubated for 30 min at 65 °C. After cooling, 500 µL chloroform:isoamyl alcohol (24:1) was added and centrifuged for 5 min at 12000 rpm. This procedure was repeated twice. The supernatant was treated with RNAse A (10 µg.µL^{-1}), at 37° C for 30 min. DNA precipitation was performed with 0.6% (v/v) of cold isopropanol. The formed pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µL of Tris-EDTA pH 8.00 (TE buffer). DNA concentration was evaluated spectrophotometrically by NanoDrop 3300 (Thermo Scientific) and the quality of the DNA was checked by electrophoresis on a 1% agarose gel stained with GelRed™ (BIOTIUM™).

Initial PCR reactions were performed using thirty-one random decamer primers from Operon Technology-USA, seventeen primers (OPAD-01, OPAD-08, OPAD-10, OPAD-17, OPAD-18, OPD-04, OPD-18, OPG-19, OPI-14, OPI-19, OPI-20, OPE-06, OPE-09, OPE-12, OPP-05, OPP-06, OPP-08) were selected on the basis of the good resolution and polymorphisms of bands. Each PCR reaction was performed in a 25 µL reaction volume containing 25 ng of DNA template, 5 µL of 5X reaction buffer, 2.5 µL of 25 mM $MgCl_2$, 0.5 µL 10 mM dNTPs, 1.25 U of Taq and 0.3 µL (10 µM) of specific primers, in a Veriti® 96-Well Thermal Cycler (Applied Biosystems™, USA). The PCR cycles followed the following conditions: 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min and extension at 72 °C for 2 min. After these cycles, the samples were submitted to a final extension step at 72 °C for 10 min. Amplification products were separated on a 2% agarose gel in TBE buffer (1X), stained with GelRed™ (BIOTIUM™), visualized under UV light and recorded using a transilluminator LPIX-TOUCH (Loccus Biotecnologia, Brazil).

2.5. Preparation of hydroalcoholic extracts

The *Tithonia diversifolia* powder was macerated with 70% ethanol, protected from light, in the solvent to powder ratio of 5/1 (v/w) for 72 h at room temperature. Thereafter, the supernatant was removed and the same powder was submitted to a new maceration with 70% ethanol. After 72 h, the supernatant was removed and the powder was macerated one more time for 72 h, totaling 3 maceration steps. Then, the resulting liquids were filtered and concentrated under a vacuum evaporator to obtain the crude hydroalcoholic extracts of *T. diversifolia* (HAE).

2.6. Phytochemical screening

2.6.1. Preliminary phytochemical prospecting

The preliminary phytochemical prospection was performed based on color reactions and precipitation in order to identify secondary metabolite groups such as alkaloids, coumarins, flavonoids, naphthoquinones, saponin, steroids, tannins and triterpenoids presents in crude HAEs from all localities: ST, VIA, MF, VIT and COL (Costa, 1982; Costa, 1986; Wagner and Bladt, 1996).

2.6.2. Total phenolic content (TPC)

Total phenolic content (TPC) was measured according to the Folin-Ciocalteau method described by Zhang et al. (2006). In a 96-well microplate, ethanol solutions of HAE 500 µg mL^{-1} (20 µL) were added to 100 µL of Folin-Ciocalteau reagent diluted in distilled water (1:10). After 5 min, 80 µL of Na_2CO_3 (7.5%) were added and the plate was left in the dark at room temperature for 2 h. The absorbance was measured