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Full Length Research Paper

Agronomic and phytochemical characterization of *Cyclanthera pedata* Schrad. cultivated in central Italy

Mario Macchia[1]*, Paola Montoro[2], Lucia Ceccarini[1], Ilaria Molfetta[1] and Cosimo Pizza[2]

[1]Dipartimento di Agronomia e Gestione dell'Agroecosistema, Università di Pisa, Via S. Michele degli Scalzi 2, 56124 Pisa, Italy.
[2]Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte don Melillo, 84084 Fisciano (SA), Italy.

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The aims of this study were to provide an agronomic and phytochemical characterization of *Cyclanthera pedata* Schrad. (Cucurbitaceae family) and assess its potential for adaptation to the soil and climate conditions of Italy. At each harvest date, characteristics of seed present in the harvested fruits were analyzed with germination tests to study the influence of temperature and light conditions. In addition, quantitative chemical analyses of O- and C- chrisine and apigenine flavon glycosides, already reported in the fruits of *C. pedata* (Montoro et al., 2001; Carbone et al., 2004) were performed by HPLC (high performance liquid chromatography) coupled with ESI-ITMS (electrospray ion trap mass spectrometry) on harvested fruits. In the trial environment *C. pedata* achieved excellent results both from an agronomic point of view and with regard to the characteristics of the main active ingredients produced.

Key words: *Cyclanthera pedata*, hypoglycemic plant, germination test, HPLC-MS, flavonoids**.**

INTRODUCTION

Diet and health are, in modern thinking, inextricably linked, forming an inseparable twofold approach. Diet is widely held to influence various bodily functions, with a preventive action against numerous pathologies. One fundamental aspect of this cultural and social phenomenon is the increasing demand for natural products, in particular those belonging to or deriving from the plant kingdom, whose properties allow them to be used for medicinal, herbalist or more generally health-promoting purposes.

Cyclanthera pedata Schrad., a species belonging to the Cucurbitaceae family, has been the subject of biological studies designed to investigate its anticholesterolemic, anti-inflammatory and hypoglycemic properties. It thus represents an example of a plant used for medicinal purposes, and can appropriately be considered within the above-described context of food plant with health-giving effects.

C. pedata Schrad. is of South American origin, where it is known by the common name of Caygua. It is thought to be native to the Andean region or "Sierra", and was cultivated by the Incas who utilized its fruits as food (Dietschy, 1953; Popenoe, 1990; Macbride, 1937; Montoro et al., 2001). A scapose therophyte species, it is an annual climbing plant with tendrils, palmate leaves, and small unisexual flowers at the leaf axilla. The fruit is an ovoid pepo (Cappelletti, 1976; Macbride, 1937; Pignatti, 1982). The nations involved in promoting the diffusion of this species are Peru, Ecuador (in particular the southern part), Bolivia, Colombia, and Venezuela; in addition, its presence in the southern part of Mexico and in the Caribbean area has also been reported (Macbride, 1937).

The fruits and seeds of *C. pedata* Schrad. are rich in cucurbitacins, which are important in the sphere of chemotaxonomy (Dinan et al., 2001). A number of studies have highlighted the presence of these plant triterpenoids (De Tommasi et al., 1996; De Tommasi et al., 1999) in fruits and seeds of this plant.

In addition flavonoids O- and C-glycosides were characterized and quantified in fruits (Montoro et al., 2001; Carbone et al., 2004).

C. pedata Schrad. grows well under a fairly wide range of environmental conditions. Optimal growth temperatures

*Corresponding author. E-mail: macchia@agr.unipi.it.

are 12 - 18°C; it tends to be day neutral but, like many Cucurbitaceae, it presents great variability. The ideal soil for this species is deep and well drained, with a pH of 6.0 - 7.0. The plant does not tolerate salinity. Its growth cycle extends for 3 - 4 months in its native environment. Sowing is performed throughout the year, using seed quantities of roughly 2 kg/ha. Seeds are sown using the dibble hole method, in double rows spaced 4 m apart, with 0.5 - 1 m spacing along the rows, giving a density of roughly 0.25 plants per surface unit. In the Peruvian regions, the crop is harvested in June - July; harvesting begins 100 days after crop establishment and continues for a duration of 45 - 60 days. Yield is roughly 7 t/ha, but yield potential is 15 - 20 t/ha. (Programa de Hortalizas, UNA La Molina, 2000).

The cultivation of *C. pedata* in Italy is not documented, and the bibliography on its agronomic, biological and reproduction characteristics is extremely scanty.

The present study represents the first step towards cultivating *C. pedata* in Italy in order to establish an extract production line based on this plant, for use in the herbalist sector. Thus the aims of this study were to provide an agronomic characterization of the species and assess its potential for adaptation to the soil and climate conditions of Italy. Additionally, seed characteristics and chemical properties of fruit obtained in the trial environment were also reported.

MATERIALS AND METHODS

Plant material

Seeds were obtained from "Gesamthochschule Kassel" of Witzenhausen University and stored under the same conditions (10°C and 50% RH) until germination testing began. Germination tests were conducted at the D.A.G.A. La.R.A.S (Dipartimento di Agronomia e Gestione dell'Agroecosistema Laboratorio di Ricerca ed Analisi sulle Sementi [Department of Agronomy and Agro-Ecosystem Management. Seed Research and Analysis Laboratory]) using 15 cm diameter Petri dishes moistened with 9 ml distilled water. The filter paper substrate was maintained moist by periodic addition of distilled water. To reduce water loss to a minimum, dishes were wrapped in transparent polyethylene bags. To avoid possible primary dormancy phenomena, seeds were pre-chilled at 5°C in dark for 7 days. After the pre-chilling treatment, seeds were transferred to a heat-controlled cabinet at alternating temperature of 20/30°C (16/8 h).

Germinated seeds were then placed in plug trays filled with peat, in a cold greenhouse in April. In May the plugs were transferred to 35 cm diameter pots having more than 5 liter capacity and filled with sand and compost mixture soil (1:2). The plants were cultivated at the Experimental Centre of Rottaia (Pisa, Central Italy), in deep silt loam soil (clay 7%, silt 34%, sand 59%, pH 8.2, organic matter 1.4%). Tillage was carried out in the autumn of 2002 and consisted of medium depth ploughing (30 cm). Seedbed preparation was conducted by a pass with a double-disking harrow and a pass with a field cultivator. Preplant fertilizer was distributed at a rate of 100 kg ha^{-1} of N (urea), 100 kg ha^{-1} of P_2O_5 (triple superphosphate) and 100 kg ha^{-1} of K_2O (potassium sulphate). After transplanting, drip irrigation was applied to ensure that planting out was successful. Irrigation was then continued throughout the plant cycle. Weeds were controlled with mechanical means.

The 15 plants thereby obtained were planted out in the open field in single rows spaced 80 cm part. Since the plant has a creeping habit, staking was provided using roughly 20 m long double netting fixed to supports that were placed at each plant.

To take scalar production into account, fruits were harvested on three successive dates: 1st August, 30th August and 23rd October 2002. Harvesting was performed manually.

Seed germination tests were conducted on harvested seed, in order to assess germination characteristics in local Italian soil and climate conditions.

Germination tests were performed at D.A.G.A. La.R.A.S using 150 mm diameter Petri dishes moistened with 9 ml thyram solution at a concentration of 0.4 g per 500 ml distilled water. The filter paper substrate was maintained moist by periodic addition of distilled water. To reduce water loss to a minimum, dishes were wrapped in transparent polyethylene bags. To study the influence of temperature on germination, seven different heat regimes [15, 20, 25, 30, 35°C, constant temperature and 15/25°C, 20/30°C (16/8 h) alternating temperature] were adopted. Germination tests were conducted both in continuous light supplied by cool white-light fluorescent lamps (Osram L18 W/20, 10 µmol photons s^{-1} m^{-2} photosynthetically active radiation) and in darkness (ISTA, 2005). Tests had a mean duration of 21 days, during which the number of germinated seeds was counted. Seeds were considered germinated when the rootlet reached the length of the seed itself. In addition, germination energy was evaluated by calculating mean germination time (MGT) using the formula of Ellis et al. (1981).

Quantitative HPLC-ESIMS analysis

Quantitation of compounds was achieved by using external standards and internal standard (naringine). Standard curves for each of the flavonoid standards were prepared by pure compounds over a concentration range of 5 - 125 µg mL^{-1} by using 5 different concentration levels and triplicate injections at each level. All the solutions were prepared by the dilution of 1 mg mL^{-1} stock solution of each standard. A quantity of 40 µg mL^{-1} of internal standard was added to calibration samples and to extract samples.

Peak areas were obtained from RICs (reconstructed ion chromatograms) relative to external standard pseudomolecular ions and peak area, obtained from RIC relative to internal standard pseudomolecular ion.

Peak area ratios between the area of the peak of each flavonoid standard and those of naringine (40 µg mL^{-1}), used as internal standard, were calculated for each concentration level and plotted against the corresponding standard concentration using weighed linear regression to generate standard curves. Software used for plotting was Xcalibur.

74.88 g of fresh fruits were fragmented and freeze-dried for 5 days, to give an amount of 3.8 g freeze-dried fruits (94.92% water), which internal standard naringin (10 mg) subsequently was added. Fruits were extracted with MeOH (ratio drug/solvent 1 g/10 mL), by ultrasound for 60 minutes at room temperature, and then macerating materials were stored at room temperature in the dark for a night. 5 g of dried fruit powder, deprived of seeds, were added with internal standard, naringin (10 mg), and extracts with MeOH and then diluted, following the steps already described.

LC-MS analyses were performed by LC/ESI/MS "on-line" using a Thermo Finnigan Spectra System HPLC (quaternary pump, degasser and autosampler) coupled with an LCQ Deca ion trap (Thermo Electron, San Josè, CA, USA), equipped with Xcalibur software for data elaboration. Analyses were performed by using a Waters Symmetry C18 column (150 × 2.1 mm i.d.; particle size 5 µm) and as mobile phase a gradient of 0.05% TFA (trifluoroacetic acid) as eluent A and acetonitrile with 0.05 % TFA as eluent B. Elution was performed, after 5 min of isocratic step at 10% of solvent B, by means of a linear gradient from 90:10 (A: B) to 60:40 (A :B)

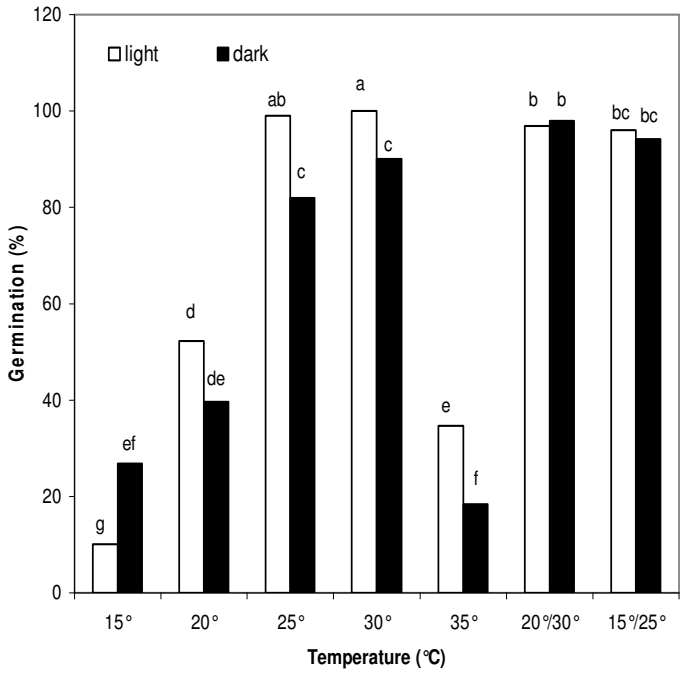

Figure 1. Germination percentages of *Cyclanthera pedata* seeds under different temperatures adopted. For each mean followed by the same letters are not significantly different at the 0.05 probability level according to LSD test.

over 40 min, then a subsequent step from 60:40 (A: B) to 5:95 (A:B) in 15 min, at a flow rate of 0.3 mL min^{-1}. The flow generated by chromatographic separation was directly injected into the electrospray ion source working at room temperature. The positive ion mode for MS and MS/MS analysis was selected.

Electrospray source worked at a temperature of 280°C, the capillary voltage was at 5 V, the spray voltage was at 5 kV and the tube lens offset was at 35 V. Nitrogen flow was set at 80% value (arbitrary units).

The analytical and quantitative method was carried out according to details reported by Carbone et al. (2004) and Montoro et al. (2005).

RESULTS AND DISCUSSION

No particular difficulties were encountered in growing *C. pedata*, as this species does not require special crop management techniques apart from proper staking and supports. From the earliest growth stages onwards, plants showed vigorous development and were successfully able to compete with weeds. *C. pedata* adapted well to the growth environment, reaching a height of roughly 370 cm within two months after planting out, which took place in May.

Fruits were harvested at three separate times: end of the months (30^{th}) of July, August and September. Maximum yield in numerical terms (108 fruits pt^{-1}) and in fruit dry weight (49 g pt^{-1}) was obtained at the end of August, with significantly different values compared to the September harvest. Production at the first harvest time was characterized by a fairly elevated number of fruits; therefore

Table 1. Principal characteristics of harvested fruits. Each mean followed by the same letter are not significantly different at the 0.01 probability level according to LSD test.

Analyzed parameter	Period of harvesting		
	July	August	September
Width (cm)	2.50 A	2.61 A	1.84 B
Length (cm)	6.48 B	7.62 A	4.74 C
Number of fruits pt^{-1}	63.54 A	108.23 A	34.15 B
Weight of fruits (d.w. g pt^{-1})	41.51 B	48.97 A	10.26 B

inferior in weight and size (above all length) compared to fruits obtained at the second harvest (Table 1). Fruits harvested at the end of August showed the greatest mean size (Table 1).

At each harvest date, yield and germination characteristics of seed present in the harvested fruits were analyzed. The mean number of ripe seeds per fruit was roughly 7, but additional incompletely formed seeds were also observed. Such a result is rather poor in comparison to mean production of 12 seeds per fruit normally achieved in the areas where the species grows natively. Unusable seeds consisted mainly of those which failed to ripen fully. The material obtained at the final harvest date was characterized by a high percentage of empty seeds.

Seed germination tests were performed on *C. pedata* seeds harvested during the first year of cultivation, in order to identify the most suitable heat and light conditions for germination. Seeds germinated under all test conditions assayed (15, 20, 25, 30, 35, 20 - 30°C and 15 - 25°C). However, highest germination percentages were achieved at continuous temperatures of 25 and 30°C (Figure 1). Furthermore, germination tests con-ducted at these two temperatures showed a significantly greater seed response under conditions of light (99 and 100% respectively) as compared to dark germination (82 and 90%). Alternating temperatures also gave good germination results (96%) and in contrast to continuous temperatures, alternating temperatures showed no significant differences attributable to presence or absence of light (Figure 1). However, evaluation of results from the entire range of germination tests in terms of the light conditions adopted (Figure 1) suggested that the overall response of *C. pedata* seeds to light is significantly positive. Thus germination percentage increased from 67 - 78% with the transition from dark germination to light-induced germination.

Seed germination energy, measured as mean germination time, showed no significant difference between presence or absence of light. Mean germination time was significantly lower for seeds germinated under alternating temperatures of 15/25°C, 20/30°C and constant temperatures of 20 and 35°C (Figure 2), as compared to the other treatments examined.

Chemical analyses performed on harvested fruits showed that flavonoids identified in the sample fruits are

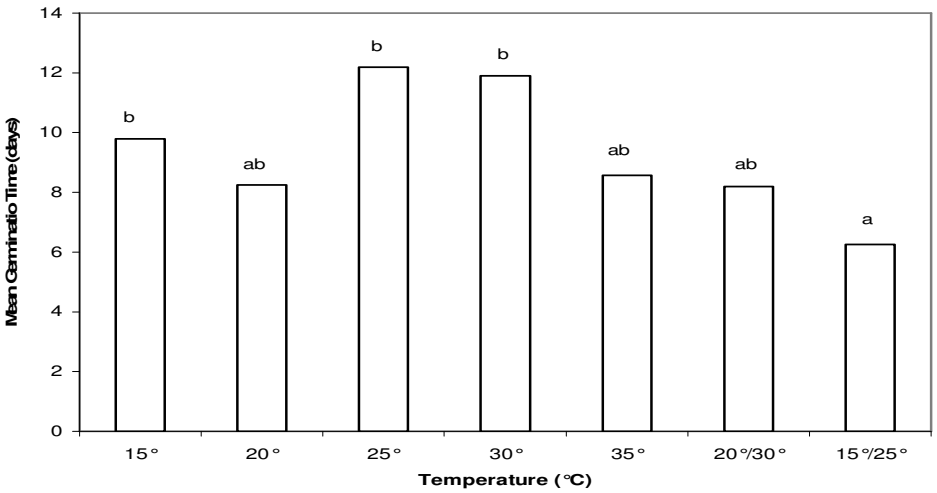

Figure 2. Mean germination time (MGT) of *C. pedata* seeds under different conditions of temperatures. Each mean followed by the same letter are not significantly different at the 0.05 probability level according to LSD test.

Table 2. Flavonoids identified in the sample of dried or dehydrated fruits produced in Italy.

Flavonoids (by LC/MS)	Freeze dried fruits		Air dried fruits	
Compound	**mg g^{-1}**	**SD**	**mg g^{-1}**	**SD**
Chrisin-6-C-fucoside7-O glycoside	0.651	0.02	0.869	0.05
Chrisin-6C-glycoside	0.038	0.00	0.676	0.05
Apigenin-6C-fucoside	0.514	0.05	0.708	0.03
Chrisin-7-O-gentiobioside	0.967	0.04	0.146	0.01
Chrisina-6-C-fucoside	0.063	0.01	0.067	0.03
Isovitexin	0.421	0.04	0.395	0.04
Chrisin-7-O-hesperidoside	0.103	0.01	0.046	0.02
Apigenin 6C- malonyl fucoside	0.908	0.02	0.078	0.001
Apigenin 6C-malonyl fucoside	1.671	0.05	0.024	0.004
Chrisin-6C-fucoside7-O malonyl glycoside	0.726	0.04	0.314	0.02
Chrisin-6-C- malonyl fucoside	1.883	0.06	0.112	0.005
Chrisin-6-C- malonyl fucoside	1.731	0.08	0.125	0.01
Total	9.676		3.561	

are consistent with literature (Montoro et al., 2001; Carbone et al., 2004) and specifically *O*- and *C*-glycosides, of chrisine and apigenine, whose sugar units may be composed of fucose, glucose and ramnose. The data shown in Table 2 indicate a greater quantity of flavonoids in freeze-dried as compared to desiccated fruits. Total concentration of such compounds in the trial samples was quantitatively greater compared to results obtained with fruits produced in the native area of *C. pedata.*

Concentration of the first 6 compounds typical of *C. pedata,* as obtained in the trial samples, was compared with concentrations recorded in analysis of fruits produced in the native growing area (Carbone et al., 2004). A lower quantity of compounds 1, 2, 5 and 6 was found in the trial samples; in particular, only very small quantities of compounds 2 and 5 were recorded in the Italian fruits. The Italian production was characterized by a good concentration of compound 4, for which is reported in literature an elevate antioxidant activity against lipid peroxidation (Montoro et al. 2001). Furthermore, although the anti-oxidant activity declined over time, it remained fairly elevated even after 120 minutes. The Italian fruits were also characterized by elevated quantities of the other 5 malonyl-derived flavonoids, three of which belong to the chrisine-6-C-fucoside group and two to the apigenine 6-C-fucoside group. Among these malonyl derivatives, the compounds 9, 11 and 12 were present in quantities greater than 1.5 mg/g. Such a concentration strongly differentiates the fruits harvested in the Italian trial area from those obtained in the native areas, where the above

mentioned compounds are present in only very small quantities.

Conclusions

The results obtained in the present study demonstrate the possibility of growing this “new species” at Italian latitudes. In the trial environment *C. pedata* achieved excellent results both from an agronomic point of view and with regard to the characteristics of the main active ingredients produced.

The possibility that species utilizable for medicinal purposes can be grown in Italy would also offer assurance concerning the safety and quality of the final product, as this would allow control over all stages of the production process. However, further study will be required on other aspects of crop management practices (fertilization, irrigation, harvest times) that may affect the quanti-qualitative characteristics of the final product.

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