Piper peltatum: Biomass and 4-Nerolidylcatechol Production

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Abstract

Piper peltatum L. is used for the treatment of inflammation, malaria, and other ailments. 4-Nerolidylcatechol (4-NC) is a valuable natural product that has important anti-inflammatory, antimalarial, and antioxidant properties. 4-NC is a component of *P. peltatum* and *P. umbellatum* extracts, which are used in cosmetics. The aim of this work was to evaluate the production of plant biomass and the production of 4-NC in roots of cultivated *P. peltatum* over a full life cycle. Seedlings were produced in a greenhouse and then transplanted. The weight of dry plant parts (leaves, stems, roots, and inflorescences); numbers of stems, leaves, and inflorescences; and the leaf-to-stem ratio were evaluated at intervals of 60 days after transplanting (DAT). Extracts were prepared using 1 : 1 ethanol–chloroform and an ultrasound bath. Roots, leaves, and inflorescences contained 4-NC according to TLC photodensitometry analysis. Quantification of 4-NC in root extracts was performed using HPLC-DAD analysis. Per-hectare production of 4-NC by roots was estimated based on quantitative HPLC analysis and biomass data. Optimal per-hectare yields of 4-NC were obtained by harvesting roots between 350 and 400 DAT. In this period, the average yield was 27 kg 4-NC per hectare. Importantly, at the time of maximal overall production of root biomass (470 DAT), there was a decrease in the production of 4-NC (23.8 kg/ha), probably due to the onset of senescence.

Key words

Piper peltatum · Piperaceae · 4-nerolidylcatechol

Piper peltatum L. (*Pothomorphe peltata* L.) and the closely related *P. umbellatum* L. (Piperaceae) share the common names caapeba and pariparoba in Brazil. Infusions of the roots and/or leaves of both species are used in the treatment of malaria [1], erisipela (a skin ailment caused by *Staphylococcus* spp.), hepatitis, and leishmaniasis [2]. Pharmacological evaluation of the extracts of *P. peltatum* has revealed important *in vivo* antimicrobial [3], analgesic [4], and antioxidant [5] activities. Extracts of *P. peltatum* and *P. umbellatum* display significant *in vitro* antimalarial activity [6, 7]. No significant mutagenic effects are associated with *P. umbellatum* extracts [8]. In addition, *P. umbellatum* displays important anti-inflammatory activity [4], especially in the case of skin exposed to excessive solar (UVB) radiation [9].

Biological activity in *P. umbellatum* and *P. peltatum* extracts has been attributed to the secondary metabolite 4-nerolidylcatechol (4-NC), which is a sesquiterpene of mixed biosynthetic origin [10]. 4-NC is present in roots, leaves, and inflorescences and has important *in vitro* and *in vivo* biological activities. For example, 4-NC inhibits the human malaria parasite *Plasmodium falciparum in vitro* [11]. It is also a potent antioxidant that displays 20 times the *in vitro* antioxidant activity of vitamin E [12]. Also, 4-NC has *in vivo* photoprotective [9, 13], *in vivo* anti-inflammatory [4], *in vitro* antifungal, *in vitro* leishmanicidal [14], *in vitro* antitrypanosomal [6], and *in vitro* cytotoxic [15] activities, among other biological activities. Interestingly, semisynthetic ether and ester derivatives of 4-NC display *in vitro* antimalarial and cytotoxic activities and greater stability than 4-NC [16].

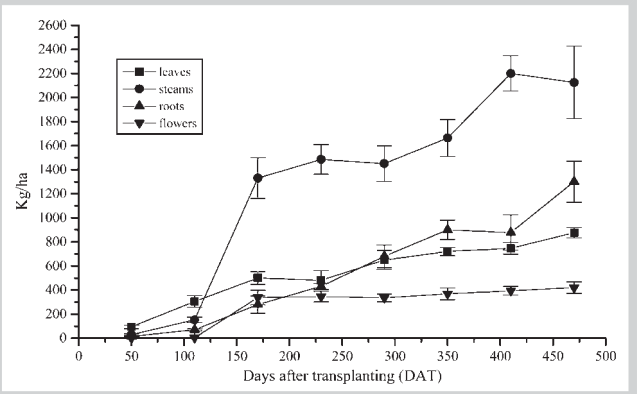

Fig. 1 Production of leaf, stem, root, and inflorescence biomass for cultivated caapeba (*Piper peltatum* L.) as a function of harvest time. Manaus, Amazonas State, Brazil, 2004–2005. Each harvest period is represented by 16 plants (4 repetitions × 4 plants).

Recently, there has been interest in the propagation of *P. peltatum* and *P. umbellatum* with the ultimate goal of evaluating the production of 4-NC. Micropropagation through direct organogenesis from the leaves of *P. umbellatum* to obtain plantlets for conventional cultivation and later extraction of 4-NC, *in vitro* propagation to obtain clones that produce 4-NC [17, 18], and evaluation of the biomass and capacity to produce 4-NC in cell suspensions [19] have been studied. The effects of indolebutyric acid (IBA), 6-benzylaminopurine (BAP), and indoleacetic acid (IAA) on root production by *P. peltatum* root and sprout cuttings and seeds also have been investigated [20]. While recent studies have demonstrated that *P. peltatum* [21] and *P. umbellatum* [22] can be readily propagated from stem cuttings or seeds and then cultivated, there are no data on the seasonal production of biomass and 4-NC by cultivated *P. peltatum*. In the present study, production of biomass and production of 4-NC by roots of *P. peltatum* were evaluated over a full cultivation cycle with the aim of establishing useful parameters for production of plant materials and 4-NC for medicinal use.

The greatest biomass production during cultivation of caapeba was observed for stems, which reached a maximum of 2200 kg/ha on the 7th harvest (400 DAT) (**Fig. 1**). The second greatest contribution to overall biomass was by roots starting at 300 DAT. By the final harvest, roots had attained an average biomass of 1300 kg/ha. Leaf biomass was greater than that of roots until 300 DAT. The vegetative phase lasted 100 DAT and then inflorescences began to form. At no time was the inflorescence biomass per hectare greater than any other plant part. At the end of the life cycle of the plants, the leaves become senescent and fall off, and seed-filled inflorescences lose weight, which reduces the

contribution of these components to the biomass. At this same phase, stems are mature and lignified and contain high percentages of cellulose.

The secondary metabolite 4-NC was determined by HPLC analysis of the ethanol–chloroform extracts of roots for each of the different harvest periods. In general, the percentage yield (m/m) of 4-NC in root extracts decreased over time. Thus, in the first harvest (50 DAT) the yield of 4-NC in root extracts was 45.3 %, while in the last harvest it was 18.3 %. In terms of overall yield per hectare, the production of 4-NC increased with the number of DAT over the first 7 harvest periods reaching a maximum of 27.4 kg/ha and decreased to 23.8 kg/ha in the last harvest period.

As stated above, infusions of roots and/or leaves of *P. peltatum* are used medicinally. However, there is no information in the literature on the chemical composition of infusions of this plant. Thus, an attempt was made to detect 4-NC in infusions prepared from dry, ground leaves (5.33 g, 30 mL 100 °C water, 15 min) and roots (5.51 g, 30 mL 100 °C water, 15 min) from the 7th harvest. Aliquots of these infusions of leaves and roots were analyzed, as was a sample of pure 4-NC in chloroform (0.54 mg/mL), under the same HPLC conditions used in the analysis of ethanol–chloroform extracts. Samples of infusions were also spiked with pure 4-NC and then analyzed for comparison. Importantly, 4-NC was detected in these relatively concentrated infusions of roots but not in those of leaves. An estimate of the concentration of 4-NC in the root infusion (10.0 g ground roots, 100 mL 100 °C water, 15 min) was ca. 8 mg/L based on semiquantitative normal-phase TLC photodensitometry. Detection of 4-NC in infusions of roots is an important finding that has implications for the medicinal use of this plant.

In this study, *P. peltata* was cultivated from February 2004 to April 2005. In this region of the Amazon, the first half of the calendar year corresponds approximately to the period of greatest rainfall. While the last harvests took place during the rainy season of 2005, plants presented clear signs of the onset of senescence (yellowed leaves, lignified stems). Although this species produces new leaves during its life cycle, the age of the plant determines the end of the life cycle. The greatest production of 4-NC per hectare by roots was in the rainy season (normally from December to May in this region) as evidenced by data from December (6th harvest) and February (7th harvest), where 24.9 kg 4-NC/ha and 27.4 kg 4-NC/ha, respectively, were recorded. Importantly, at the time of maximal overall root production (8th harvest), there was a decrease in the production of 4-NC (23.8 kg/ha). Thus, optimal per-hectare yields of 4-nerolidylcatechol from *P. peltata* roots can be obtained by harvesting between 350 and 400 DAT, when the average yield is 27 kg 4-NC/ha (**Fig. 2**).

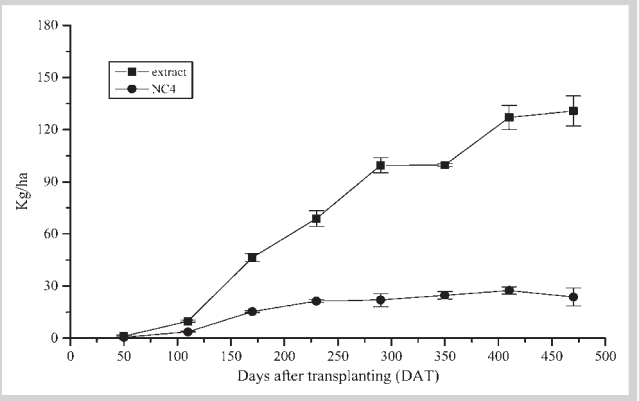

Fig. 2 Production of ethanol–chloroform extract and 4-NC (determined by HPLC-UV analysis) by roots of cultivated caapeba (*Piper peltatum* L.) as a function of harvest time. Manaus, Amazonas State, Brazil, 2004–2005.

Materials and Methods

Plant materials

Seeds were obtained from a specimen of *P. peltatum* (INPA Herbarium, voucher no. 210168) in the CPPN/INPA medicinal plant collection. Germination took place in black polyethylene bags containing organic substrate. Seedlings matured in a greenhouse at Embrapa Amazonia Ocidental (Manaus, Amazonas State, Brazil). The experimental area was plowed, grated, and corrected with 4 ton/ha of limestone. In December 2003, 50-day-old seedlings having an average height of 20 cm and 3–5 leaves each were transplanted into experimental lots. The experimental design was based on random blocks with four repetitions and four plants per repetition (16 plants per block and four plants in the useful area of each block). Plant spacing was 1.0 × 1.0 m. In all, eight harvests were performed at 50, 110, 170, 230, 290, 350, 410, and 470 days after transplanting (DAT). For each harvest, the weight of dry plant parts (leaves, stems, roots, and inflorescences); numbers of stems, leaves, and inflorescences; and the leaf-to-stem ratio were evaluated. Plant parts were separated and dried in an oven with forced circulation and renovation of air at a temperature of 45 °C until a constant weight was attained.

Estimation of per-hectare production of 4-NC

Per-hectare production of 4-NC (kg/ha) by *P. peltatum* roots was estimated by multiplying root biomass, the yield of root extract, and the concentration of 4-NC in the root extract for each harvest period.

Chemistry

4-NC was isolated from *P. peltatum* roots and purified as described previously [11]. Its identity was ascertained based on its spectral properties, which were identical to those reported in the literature [23,24]. The purity of the sample used in the present analysis should be considered to be > 95 % based on TLC, HPLC-DAD, and NMR analyses. In preliminary work, different solvents, extraction times, and methods (maceration, reflux, soxhlet, and ultrasound) were evaluated qualitatively using normal-phase and reversed-phase TLC. Thus, an extraction method was established that provided extracts enriched in 4-NC, that provided good TLC and HPLC resolution of 4-NC, and that left minimal amounts of unextracted 4-NC in the residual plant material after extraction. Determinations of 4-NC began with duplicate extractions of roots. Thus, 5.00 g of finely powdered roots were extracted with $CHCl_3$–EtOH (1 : 1, 150 mL) in an ultrasound bath for 15 min. The solvents were removed by filtration, and the residual plant material was further extracted two times using the same procedure. The combined solvents from these extractions were concentrated under vacuum using a bath temperature of 30–40 °C and then totally evaporated in preweighed glass vials and freeze-dried. The yield of extract was calculated as the average of two determinations.

4-NC external calibration

A 1.00 mg/mL stock solution of 4-NC in acetonitrile (ACN) was prepared. Dilutions having concentrations of 0.10, 0.20, 0.30, 0.50, and 0.70 mg/mL were prepared from the 4-NC stock solution in ACN. Diluted 4-NC solutions were analyzed in triplicate on an HPLC apparatus using the conditions described below.

HPLC apparatus and conditions of operation

The following equipment was used for HPLC: A Shimadzu SCL-10AVP system controller; the processing software program CLASS VP; a DGU-14A degasser; dual LC-6AD pumps; a 10AF autosampler; an SPD-M20 diode-array detector (282 nm); and a LichroCART (250–4 mm Lichrospher, 100 RP-18, 5 µm; Merck) column [mobile phase: ACN (Merck; Lichrosolv)–deionized water (MiliQ) 25 : 75 (0 min) to 100% ACN (18 min), 100% ACN (20 min); flow rate: 1 mL/min; injection volume: 5 µL]. Under these conditions, the 4-NC peak had a retention time of 11.4 min. Where y is the absorbance and x is the concentration of 4-NC in mg/mL, a calibration curve having good linearity was obtained through linear regression analysis: $y = 2.10^6 x + 1.98 \times 10^3$, $r^2 = 0.999$.

Determination of the limits of detection and quantification

The limits of detection (LOD) and the limits of quantification (LOQ) were defined as being those concentrations providing 4-NC peak heights 3 times (S/N = 3) and 5 times (S/N = 5) those of noise and were established as 5 and 10 µg/mL, respectively, under the conditions used for analysis described above.

Analysis of extracts

Each root extract (10.0 mg) was dissolved in a few drops of CH_2Cl_2 or $CHCl_3$ using an ultrasound bath, and the resulting solution was treated by solid-phase extraction in a Sep-pak RP-18 (Supelco) cartridge (previously washed with acetone and ACN). Elution from the cartridge was performed with ACN (8 mL). The ACN fraction of each extract was diluted to 10.0 mL. A 1.0-mL aliquot of this solution was filtered in an IC Millex-LG 0.2-µm cartridge into an amber sample vial and was analyzed by HPLC as described above for 4-NC with monitoring at 200, 254, 282, 365, and 400 nm. Each extract solution was analyzed by HPLC in triplicate. In HPLC analyses of root extracts, the 4-NC peak presented retention times in the range of 11.3 to 11.9 min.

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