Metabolic response of *Adenocalymma peregrinum* during regeneration of the aerial parts

Vanessa Samudio dos Santos,[1] Maria Rita Marques,[1] Dayana Rubio Gouvea,[2] Norberto Peporine Lopes,[2] Vespasiano Borges de Paiva Neto,[3] Carlos Alexandre Carollo[1]

[1]Center of Biological and Health Sciences, Federal University of Mato Grosso do Sul, Campo Grande; [2]NPPNS-School of Phamarceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto; [3]Campus of Chapadão do Sul - CPCS, Federal University of Mato Grosso do Sul, Chapadão do Sul, Brazil

Abstract

Adenocalymma peregrinum, popularly known as *ciganinha*, is an aggressive pasture invader. This species has a vegetative propagation mechanism that includes aerial latent buds and subterranean stalks activated by cuts and other lesions. In the present study, we evaluated the levels of cytokinins and secondary metabolites in *A. peregrinum* during regeneration of the aerial part. Plants used in this experiment were established in the field and were cut at intervals of five days until the 25th day. The regeneration of the cut plants started on the 10th day after the first cut. Among observed compounds, only kinetin, allantoin and the iridoide 6-β-hydroxyipolamiide were detected at adequate levels for comparative evaluation. The maximum levels of these compounds coincided with the beginning of regeneration in these plants, indicating the involvement of these compounds in the regeneration process.

Introduction

Environments altered by humans facilitate invasions by wild or exotic species, resulting in economic and environmental losses.[1,2] *Adenocalymma peregrinum* (Miers) L.G. Lohmann (Bignoniaceae) (popularly known as *ciganinha*), is characterized by the ability to disperse rapidly following cutting by animals or fires. This mechanism is made possible by vegetative propagation that includes aerial latent buds and subterranean stalks.[3]

The regeneration of *A. peregrinum* is efficient and is mediated by biochemical mechanisms specifically developed to counter environmental stresses,[4] thereby enabling this species to invade pastures and cause serious damages to local husbandry.

In plants incapable of fixating atmospheric nitrogen, including some species of the Bignoniaceae family, compounds similar to allantoin have been shown to be adequate alternative sources of nitrogen.[5] Grassi *et al.*[6] isolated large amounts of allantoin from *A. peregrinum* and suggested that this ureide could be used to establish a competitive advantage over other species.

Cytokinins have a key role in the differentiation and regeneration of plants, namely, by inducing cellular division, proliferation and morphogenesis of the aerial part.[7] This phenomenon may also be related to adaptation strategies and the rapid development of *A. peregrinum*. To analyze compounds present at very low levels, such as plant hormones, previous literature has described the use of methods based on liquid chromatography (*e.g.*, studies by Peres *et al.*[8] and Zhang *et al.*[9]) and liquid chromatography combined with mass spectrometry (HPLC-MS) using electrospray ionization (*e.g.,* a study by De Vos *et al.*).[10] These methods have been used in several plant hormone studies, allowing simultaneous quantification of several compounds.[11]

Therefore, the objective of this study was to evaluate the levels of cytokinins and secondary metabolites in *A. peregrinum* during regeneration of the aerial parts.

Materials and Methods

Material collection for analysis

Plant material was collected in Brazil, Campo Grande, Mato Grosso do Sul (20°37'56,30"S; 54°34'14,00"W). For the botanical identification, fertile material of *A. peregrinum* was herborized, identified and deposited in the CGMS herbarium of UFMS under number 33487.

The aerial parts of adult plants established in the field were used for analysis. The aerial parts were removed leaving 10 cm of above ground material. Stalk samples collected for analysis left 5 cm of the superior part near the previously made cut. The stalk samples were collected at 0, 5, 10, 15, 20 and 25 days after the removal of the aerial part, and each group consisted of seven specimens. The samples were frozen in liquid nitrogen at the moment of collection and stored at –80°C until use.

Extraction and analytical methods for the evaluation of cytokinins and secondary metabolites

The following HPLC-grade solvents were used in the extraction and resolubilization tests: methanol, ethanol, N-propanol and formic acid. The analytical method was optimized for the comparative evaluation of the cytokinins and secondary metabolites present in the samples (data not shown).

For metabolite extractions, 50 mg of plant material was added to 2 mL of ethanol/H_2O/formic acid (9:1:0.1 v/v/v). The samples were extracted for 15 minutes in an ultrasonic bath. Following extraction, 1.5 mL of the supernatant was removed and added to 500 µL of hexane. After 10 minutes, the hexane was removed, and the supernatant was dried in a speed vacuum

Correspondence: Carlos Alexandre Carollo, Center of Biological and Health Sciences, Federal University of Mato Grosso do Sul, 79070-900 Campo Grande, MS, Brazil.
Tel.: +55.067.3345.7366 - Fax: +55.067.3345.7305.
E-mail: carlos.carollo@ufms.br

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Contributions: VSS running laboratory and experimental work; DRG and NPL conducted the analytical studies; VSS and CAC analyzed data and drafted the paper; MRM, VBPN and CAC designed the study and supervised the laboratory work; MRM, NPL, VBPN, CAC contributed to critical reading of the manuscript.

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concentrator for 10 hours at a temperature of 45°C. Resolubilization of the sample was achieved by adding 300 µL of the mobile phase, comprised of acetonitrile/H_2O/formic acid (1:1:0.1 v/v/v). All of the extractions were performed in triplicate, and the injection volume was 5 µL.

For confirmation of the compounds, we used high resolution data obtained with the UFLC Shimadzu equipment (pump model LC 20 AD, automatic injector SIL 20 A HT, CBM 20A controller, DAD detector model SPD-M 20A) coupled to a UltroTOF-Q hybrid high-resolution mass spectrometer (Bruker Daltonics Billerica, MA, USA) operating under negative or positive ionization. For chromatographic separation, a C18 column (25×4.6 cm, 5 micrometers, Sigma Aldrich) was used with a pre-column of the same material. For the comparative evaluation of the compounds, low resolution analyses were performed on an ACQUITY UPLC-DAD-MS/MS Waters system (column: ACQUITY 1.7 µm EBH (*Ethylene Bridged Hybrid*) C18, with dimensions of 2.1×50 mm).

The mobile phase was comprised of ultrapure water (Milli-Q, Millipore®) (phase A) and acetonitrile (phase B), both containing 0.1% formic acid. The temperature of the column was held at 40°C, and the samples were conditioned to 20°C. The flow rate of the mobile phase was 0.3 mL/min, and the injection volumes varied from 2 to 5 µL, depending on the concentration of the sample to be analyzed. Samples were filtered through disposable membranes with a 13 mm diameter and 0.22 µm pores. The gradients of mobile phase (% phase B) were as follows: 3% for 5 min. followed by 4% for 3 min., 5% for 1 min., 100% for 2.50 min. and 3% for 2.10 min.

Data acquisition and processing were performed using DataAnalysis 4.0 software for high resolution analysis and MassLynx 4.1.5 for low resolution analysis. The analyses monitored allantoin, 6β-hydroxyipolamiide and seven cytokinins: isopentenyladenine, kinetin, benzyladenine, isopentenyladenosine, zeatin ribose, dihydrozeatin ribose and zeatin-9-glucoside.

Data analysis

The data were submitted to statistical analysis by an ANOVA test followed by a Tukey test. The statistical analyses were performed using Minitab 16.

Results

The compound of mass *m/z* 405.1886 detected in the analysis was identified as glycosylated iridoid 6β-hydroxyipolamiide. This iridoid has previously been isolated from leaf extracts of *A. peregrinum*.[6] Due to the lack of data pertaining to the fragments generated by impact collision of electrons of this compound, we proposed a fragmentation pattern of this iridoid in this study (Figure 1). The initial compound undergoes dehydration at the source in position 5 and then fragments, losing the glucopyranoside group and thus generating the peak at *m/z* 193.0495. This compound then either dehydrates, generating the peak at *m/z* 175.0390, or loses the *O*-methyl group, forming a fragment at *m/z* 165.0549.

The regeneration of *A. peregrinum* was initiated 10 days after cutting, and by the 25th day, total recomposition of the aerial parts was evident.

Nine compounds, with the fragments and retention times described in Table 1, were detected.

Cytokinins were confirmed based on a study by Bartok *et al.*[12] In the UPLC-DAD-MS/MS analysis, the concentrations of some cytokinins were below the quantification level of the equipment (signal/noise less than 10 times). Thus, these compounds were only classified as present or absent in the experiments. Only allantoin, kinetin and 6β-hydroxyipolamiide were detected at adequate levels for comparative evaluation.

The plants whose aerial parts were removed in the field showed significant differences in relation to the concentration of kinetin between the 5th and 15th day after removal of the aerial part. Maximum concentrations of allantoin and 6β-hydroxyipolamiide coincided with the start of the regeneration process in the plants, but statistical analyses (P-value of less than 0.05) did not exhibit significant differences among the regeneration periods of the aerial parts (Figure 2).

Through mass spectrometric analysis, we observed a higher recruitment of kinetin relative to allatoin and 6β-hydroxyipolamiide during the regeneration period of the aerial part.

Discussion

The compound 6β-hydroxyipolamiide has an important role in decreasing the growth rate of many generalist herbivore insects by inhibiting their feeding.[13] Therefore, this compound could provide additional protection for the species during their recovery.

Natural as well as anthropogenic disturbances to the aerial parts of plants stimulate vegetative growth.[14] For this growth and for the formation of new tissues to occur, cellular divisions stimulated by plant hormones are

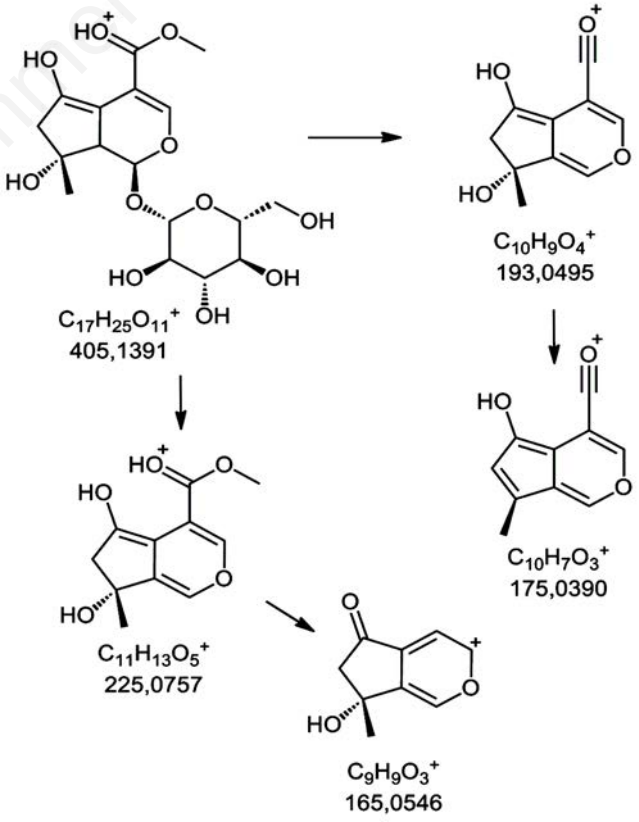

Figure 1. Proposed fragmentation of 6β-hydroxyipolamiide.

Table 1. Compounds, abbreviations, molecular masses, retention times and compound fragments observed by mass spectrometry.

Common name	Abbreviation	Molecular mass	Retention time (min)	Ion product (*m/z*)	
Allantoin	ALN	159.0512	0.50	116	-
Zeatin-9-glucoside	[9G]Z	382.1721	0.52	220	136
Zeatin ribose	[9R]Z	352.1615	0.58	220	136
Dihydrozeatin ribose	(diH)[9R]Z	354.1771	1.65	222	136
Isopentenyladenine	iP	204.1243	3.67	136	119
Kinetin	KIN	216.0879	4.79	148	81
Benzyladenine	BAP	226.1087	6.24	91	-
6β-hydroxyipolamiide	-	405.1385	6.41	193	175
Isopentenyladenosine	[9R]iP	336.1666	9.04	136	204

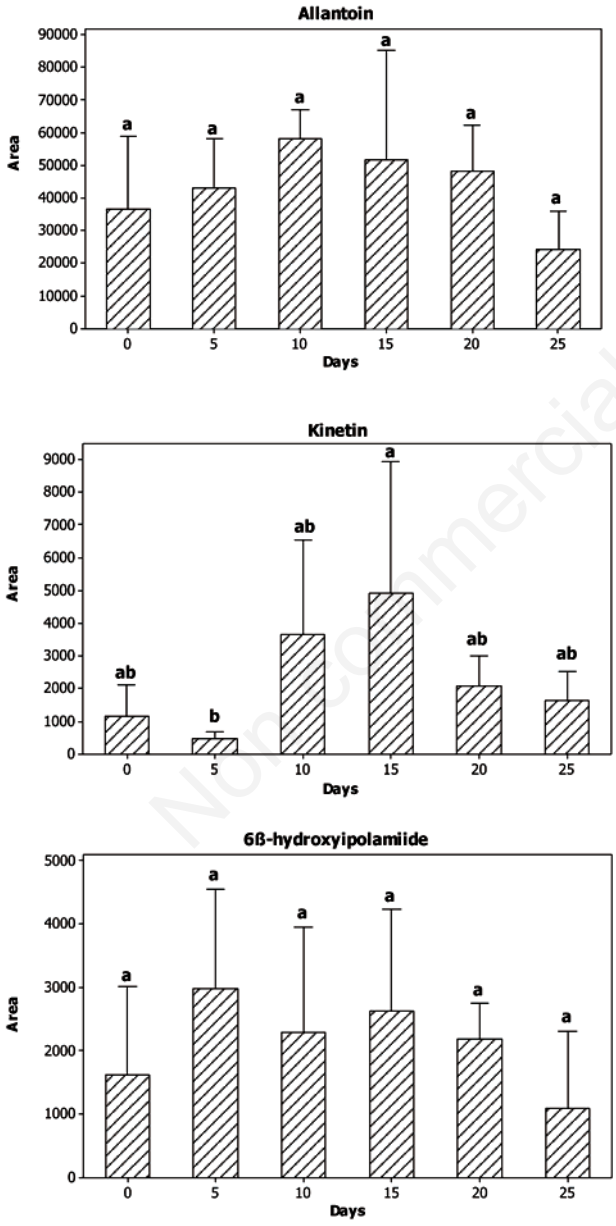

Figure 2. Areas of the peaks corresponding to allantoin, kinetin and 6 -hydroxyipolamiide in the HPLC/MS chromatogram of the stalk samples of *Adenocalymma peregrinum* at different periods after removal of the aerial parts of plants established in the field. Same letters indicate values that do not differ from each other by the Tukey test with a 5% probability of error.

necessary.[7]

The removal of the aerial part of plants stimulated rapid regeneration. During this period we observed a higher concentration of kinetin (Figure 2), which likely contributed to the recomposition of the aerial part by inducing the occurrence of cellular divisions.

The concentrations of cytokinins in this species were extremely low, confirming the concentration data of these hormones in plants reported by Peres *et al*.[15]

In a study performed by Lopes *et al.*[16] on sprouting papayas with different cut heights of the aerial parts (20, 25 and 35 cm from the plant's stem), a higher sprouting performance and bud size were observed in the plants cut 20 cm from the stem. The lower cut height promoted a higher proximity between the aerial part and the radicular system, causing rapid translocation of these compounds and elevated concentrations of cytokinins at the cut location in a shorter period of time. This effect likely explains the intensity and velocity of regrowth of plants considered to be invaders. At the moment they suffer a severe cut, these plants activate a mechanism of translocation of cytokinins to induce cellular division at the cut location for tissue regeneration.

In a study by Lopes *et al*.[17] the methodology of cutting aerial branches of *A. peregrinum* already established in the field was used with the objective of comparing the growth rate and the concentration of ureides between plants with aerial cuts approximately 10 cm above ground and a control group (no cut). It was shown that in *A. peregrinum*, there was a translocation of a large quantity of ureides from the subterraneous stalk to the aerial part following the mechanical cut, in addition to an increase in concentration of these ureides some months after the cut, suggesting that these compounds contributed to the production of new plant tissues.

The studies analyzing the concentrations of ureides in seedlings of *A. peregrinum* have also confirmed the hypothesis established by Grassi *et al.*[6] suggesting that a species produces and stores ureides in both the roots and the rhizome, and these are transported to other tissues of the plant; hence, this mechanism gives the species an advantage over other species, allowing it to establish as an invasive species.

The results of this study confirm that the damage caused to plants by disturbances in the plant tissues favors biochemical responses, such as those reported by Vázquez-Flota *et al.*[18] and that these mechanical damages can induce increases in concentrations of certain compounds in the plants, as in the case of kinetin. This increase is likely linked to the production of new tissues for the regeneration of the aerial part of the affected plant.

Conclusions

The tissue regeneration of *A. peregrinum* was rapidly initiated on the 10th day after removal of the aerial part. The comparative analysis of kinetin, allantoin and 6β-hydroxyipolamiide demonstrated that kinetin has a clear role in the regeneration of the aerial part of *A. peregrinum,* as the peak of this compound coincided with the beginning of regeneration of the aerial part in this species. In contrast, despite the observed increases in the concentrations of allantoin and 6β-hydroxyipolamiide, no significant rises were observed during the regeneration period.

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