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Allelopathic effects of *Brachiaria ruziziensis* and aconitic acid on *Ipomoea triloba* weed

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ABSTRACT

This study investigated the allelopathic potential of both aqueous fraction of *Bracharia ruziziensis* L. straws and *trans*-aconitic acid, a component of aqueous fraction on growth and physiological processes of the weed *Ipomoea triloba* L. Both aqueous fraction and aconitic acid at 250-2000 ppm concentrations influenced the germination and growth of *I. triloba* and caused similar changes in the respiratory activity of primary roots. They reduced KCN-sensitive respiration and increased the KCN-insensitive respiration. The highest concentration of both aqueous fraction and *trans*-aconitic acid increased the malondialdehyde and conjugated diene content in the primary roots of seedlings. The oxygen consumption from citrate oxidation in mitochondria isolated from primary roots was not affected. Thus, the water soluble compounds of *B. ruziziensis* were phytotoxic to *I. triloba*, inducing perturbations in respiratory activity and lipid peroxidation. Although *trans*-aconitic acid exerted similar effects to the aqueous fraction, it is not the main compound responsible for the effects of aqueous fraction in *I. triloba*, because its content is very little in this fraction.

Keywords: Aconitic acid, *Brachiaria ruziziensis* L., germination, *Ipomoea triloba* L., oxidative stress, respiration.

INTRODUCTION

The increasing concern about the environmental protection has led to the development of biologically based methods for weed control to minimise the hazardous effects of herbicides and pesticides. The selection of crop species with strong allelopathic potential may aid in development of sustainable weed management (15,37,48). Sorghum (*Sorgum bicolor* L.) is commonly used as cover crop in conservation tillage systems in several regions of the world (16,42). Sorghum residues reduces 90% weed biomass in no-till summer-planted soybean (47). Sunflower (*Helianthus annuus* L.) residues also reduces the density of several weeds (3,19). Additionally, alfalfa (*Medicago sativa* L.), rice (*Oryza sativa* L.), buckwheat (*Fagopyrum esculentum* L.) and wheat (*Triticum aestivum* L.) are also allelopathic to weeds (1,6,49).

There is also some evidence that tropical forage grasses, including the *Brachiaria* species, suppresses the weed emergence (27,31,40). Soybean and *Brachiaria* in rotation or oversowing are frequently used in crop-livestock integration in Mato Grosso cerrado and Paraná States in Brazil. Voll *et al.* (43,46) have suggested that the reduction in seed banks

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of *Commelina benghalensis* L. in areas infested with *Brachiaria plantaginea* L. is due to presence of soluble compounds (Ferulic, aconitic acids) released from the plant extracts. They also found an inverse correlation between the inhibitory actions of *B. plantaginea* extracts and pure aconitic acid on both the germination of *C. benghalensis* and the growth of endophytic fungi (43,46). The aconitic acid (2.5 mM) inhibits the growth of other weed species, including wild poinsettia (*Euphorbia heterophylla* L.), morning glory (*Ipomoea grandifolia* L.), prickly sida (*Sida rhombifolia* L.) and beggarticks (*Bidens pilosa* L.)(45).

Seed germination and the initial seedling growth are energy-demanding processes. When quiescent dry seeds imbibe water, their oxygen uptake increases and the mitochondrial energy metabolism becomes very active, initiating macromolecule biosynthesis and solute transport, which are required to complete the germination and sustain the early stages of growth. The reactivation of mitochondrial metabolism also provides an important source of reactive oxygen species (ROS) (35). Plants contain an antioxidant defence system that can efficiently destroy the superoxide radicals and hydrogen peroxide (29), including soluble antioxidant compounds (i.e. ascorbic acid, vitamin E and glutathione) and anti-oxidant enzymes (14,29,30). Perturbations in either mitochondrial metabolism or the balance between the ROS-producing and ROS-scavenging processes could be a mechanism of phytotoxicity during the seed germination and initial seedling growth. In fact, ROS-induced oxidative stress is suggested as the mode of action of allelochemicals such as coumarin (32), 2-benzoxazolinone (5) and oxygenated monoterpenes (28).

This study aimed to understand the mechanism of allelopathic action of *Brachiaria ruziziensis* L. grass species used in Brazilian agriculture (4,39). The effects of an aqueous fraction of *B. ruziziensis* leaves and stems (straw) and of *trans*-aconitic acid (an organic acid present in aqueous faction) were determined on the seed germination, initial seedling growth and physiological parameters of *Ipomoea triloba* L, a weed of soybean (21).

MATERIAL AND METHODS

Trans-Aconitic acid 98%, potassium cyanide (KCN), trichloroacetic acid (TCA) and 2-thiobarbituric acid 98% (TBA) were purchased from Sigma Chemical Co. (St. Louis, USA).

B. ruziziensis was grown in open field at Experimental Research Farm, University of Maringá, Iguatemi district, Paraná State, Brazil (S 23°21; W 52° 04′′) in November 2007. The soil physico-chemical properties were: pH (6.9), sand (86%), silt (3%), clay (11%) and organic carbon (6.08 g dm^{-3}). Seeds of *B. ruziziensis* (Germain & Evrard) and *I. triloba* were purchased from a commercial supplier (Cosmos Agrícola Produtos e Serviços Rurais Ltda, Brazil). The seeds were stored in a desiccator and cooled. The plant shoots were harvested from 90 to 100 days after emergence. The plant materials were oven dried at 40°C and chopped into small pieces.

I. Preparation of extracts from *B. ruziziensis*

Ground dried materials (nearly 1.00 kg) were extracted with methanol to obtain the crude methanol extract (66.7 g). The crude methanol extract was dissolved in a mixture

of MeOH:H_2O (1:1 v/v) and sequentially partitioned with solvents of different polarities (3 × 150 mL). The solvents were used in following order: hexane, dichloromethane, ethyl acetate and butanol. The aqueous fraction (18.37 g) remained after the butanol partition, was the only fraction used in this study. The aqueous fraction was dissolved in distilled water and at final concentrations of 250, 500, 1000 and 2000 ppm. These fractions were prepared and stored in amber glass, capped and kept in refrigerator.

II. Determination of organic acids in the aqueous fractions

HPLC analyses to quantify the organic acids were conducted with a Shimadzu LC-20AT (Kyoto, Japan) and a SPD-M20A prominence photo-diode array (PDA) detector. Separation was performed on a Shimadzu C18 column (250 mm × 4.6 mm) with a 5 µm particle size (Kyoto, Japan). A Shimpack G-ODS (4) (Shimadzu) C18 guard column was used in-line prior to the analytical column.

All data were acquired and processed with LC solution chromatography software (Shimadzu) with PDA acquisition from 190 to 800 nm. PDA output at 210 nm was used for the quantification of organic acids. A positive identification of each organic acid was accomplished by comparing standard retention time and PDA peak spectral analyses with the samples. The amounts of standards injected in 10 µL were the following: L-lactate (900 nmol); 2-oxoglutarate (20 nmol); citrate (300 nmol); succinate (900 nmol); fumarate (10 nmol); and *trans*-aconitate (10 nmol). To determine the organic acids in the aqueous extracts of *B. ruziziensis*, 10 µL of a solution of 100 mg mL^{-1} was injected.

The mobile phase consisted of 3.5 mM H_3PO_4 at a flow rate of 1 mL min^{-1}. The system was equilibrated with 30 column volumes of each new mobile phase composition prior to four injections of a mixed organic acid solution. Separation was achieved at an ambient temperature of 25 ± 2ºC. The limit of detection (LOD) was defined as a ratio of 3 for signal to noise (S/N), and all values reported for LOD are based on the pick area.

III. Seed germination and growth tests

I. triloba seeds were chemical scarified with sulphuric acid as per Huang and Hsiao (22). Briefly, 100 seeds were immersed in sulphuric acid (98% 36N) for 5 min. Then 1 L distillated water was added to the solution of seeds. The seeds were removed and washed five times in distilled water. The seeds were placed on double sheets of germination paper in plastic germination boxes (gerbox) (110 mm × 110 mm) and moistened with 8 mL distilled water (control), *trans*-aconitic acid solution or an aqueous fraction of *B. ruziziensis* (concentration range of 250 - 2000 ppm). *Trans*-aconitic acid was dissolved in distilled water. Each treatment was applied to 5-plates (replicates), and each replicate consisted of 50-seeds distributed over a gerbox. The boxes were placed in a growth chamber [8 h light (230 µmol.m^{-2} s^{-1} photon flux) at 30ºC and 16 h dark at 20ºC]. A seed was considered as germinated when the radicle was 2.0 mm or longer (16). The seeds that germinated at 12, 24, 36 and 48 h were selected for growth tests. The seedlings were removed, dried on filter paper, their hypocotyls and primary roots were excised to measure their length and fresh weight. The dry weight was evaluated after incubation of the fresh material at 80ºC for 24 h.

The mean germination time was calculated by the Equation 1 (24):

$$\bar{t} = \sum n_i t_i / \sum n_i \qquad \text{(I)}$$

Where, $\bar{t}$: Mean germination time, n_i: Number of germinated seeds between the times t_{i-1} and t_i

The speed of germination was calculated by the Equation 2 (11):

$$S = (N_1 / T_1) + (N_2 – N_1) \times 1/2 + (N_3 – N_2) \times 1/3 + ... (N_n - N_{n-1}) \times 1/n \quad \text{(II)}$$

Where, S: Speed of germination, N: Proportion of germinated seeds obtained in the first (T_1), second (T_2), third (T_3)..., (n-1) h.

The speed of accumulated germination was calculated by the Equation 3 (11):

$$AS = (N_1 / T_1) + (N_2 / T_2) + (N_3 / T_3) + ... (N_n /n) \quad \text{(III)}$$

Where, S: Speed of accumulated germination, N: Proportion of germinated seeds on time (T_1), time (T_2), time (T_3)..., h.

VI. Respiration of excised root apices

To measure the respiratory activity, the primary roots were removed from imbibed seeds or seedlings 36 h after incubation and the onset of seed imbibition in distilled water (control), *trans*-aconitic acid solution and aqueous fraction of *B. ruziziensis* (250 - 2000 ppm concentrations). The oxygen consumption of roots from *I. triloba* seedlings was measured polarographically at 25ºC using a Clark-type electrode positioned in a closed plexiglass chamber. Primary roots were cut into 5-10 mm long segments and were weighed. After weighing, the samples were immediately placed in the oxygen electrode vessel with 2 mL of nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 27 µM $FeCl_3$, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$ and 20 µM NaH_2PO_4 (10,23). To determine the contributions of mitochondrial cytochrome oxidase (COX; KCN-sensitive respiration), alternative oxidase (AOX) and the extramitochondrial oxidases (KCN-insensitive respiration) to O_2 uptake, 250 µM KCN was added to the reaction medium. Oxygen uptake was monitored for 12-15 min. Oxygen uptake rates were calculated from the polarographic records using an initial concentration of dissolved oxygen (240 µM) at 25°C (17).

VII. Isolation of mitochondria from primary roots and measurements of respiration from citrate oxidation

Mitochondria were isolated from the primary roots of seedlings grown for 72 h. The primary roots of nearly 2.500 seedlings (approx 25 g fresh material) were excised, cut into 10 mm long segments and placed in 60 mL of cold extraction medium [0.4 M mannitol, 50 mM Tris-HCl (pH 7.2), 1.0 mM EDTA, 1.0 mM $MgCl_2$, 0.1% cysteine (w/v) and 0.5% (w/v) fatty acid-free bovine serum albumin]. The material was subsequently homogenised using a van crusher for 5 s. The homogenate was filtered through several layers of cheesecloth and the filtrate was centrifuged at 1.400 × g for 10 min after adjusting the pH to 7.2 with KOH. The supernatant was centrifuged at 20.000 × g for 10 min. The mitochondrial pellet was suspended in 2.0 mL medium [0.3 M mannitol, 1.0 mM

EDTA, 20 mM HEPES (pH 7.2) and 0.2% (w/v) fatty acid-free bovine serum albumin]. All operations were done at 0-4 °C (23).

Oxygen uptake was measured at 25°C using a Clark-type electrode positioned in a closed plexiglass chamber. The reaction medium contained 0.4 M mannitol, 5.0 mM KH_2PO_4, 5.0 mM $MgCl_2$, 1.0% fatty acid-free bovine serum albumin (w/v), 10 mM Tris-HCl (pH 7.2) and mitochondrial protein (0.26-0.48 mg protein). Respiration was initiated by adding 10 mM citrate. Oxygen uptake was monitored polarographically for 5 min (23).

VIII. Lipoxygenase activity measurement

Lipoxygenase activity was assayed in the root extracts from seedlings grown for 36 h in distilled water (control) or in the presence of either a *trans*-aconitic acid solution or an aqueous fraction of *B. ruziziensis* (concentration range of 250 - 2000 ppm). The roots (about 200 mg fresh weight) were weighed, transferred to a mortar and thoroughly mixed with 2.0 mL of a cold 50 mM K-phosphate (pH 7.0) solution containing 0.1% Triton X-100 (v/v). Extracts were then centrifuged for 10 min at 12.000 × g and 5ºC (38). Lipoxygenase was measured polarographically with a Clark-type oxygen electrode. The reaction medium contained 100 mM K-phosphate (pH 7.0) and 100 µL of enzyme extract. The reaction was initiated by adding linolenic acid (3.0 mM final concentration) dissolved in Tween 20. Oxygen uptake was monitored for 3-5 min, and the enzyme activity was expressed as nmol O_2 min^{-1} (mg protein)$^{-1}$. Controls were run to exclude effects of solvent.

IX. Lipid peroxidation products

Primary roots were excised from seedlings grown for 36 h in absence or presence of *trans*-aconitic acid solution or an aqueous fraction of *B. ruziziensis* (concentrations ranging from 250 - 2000 ppm). The level of lipid peroxidation in root extracts was measured in terms of malondialdehyde (MDA) and conjugated diene contents. About 400 mg excised roots were homogenised in 4.0 mL of 96% (v/v) ethanol. The content of malondialdehyde was assayed in 3.0 mL homogenate. An equal volume of 10% TCA containing 0.5% TBA was added to the homogenate, which was then heated at 95ºC for 30 min and then quickly cooled on ice. The absorbance of supernatant was read at 532 nm after centrifugation at 10.000 × g for 10 min. The correction for non-specific turbidity was made by subtracting the absorbance at 600 nm from that at 532 nm. The concentration of MDA was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ (20) and expressed as µmol (g root fresh weight)$^{-1}$.

To measure the conjugated dienes, 1.0 mL aliquot of homogenate was mixed with 0.7 mL of 96% ethanol and centrifuged at 10.000 × g for 10 min. The absorbance of supernatant was read at 234 nm, from which the non-specific absorbance at 500 nm was subtracted. The concentration of conjugated dienes was calculated using the extinction coefficient of 2.65×10^4 $M^{-1}cm^{-1}$ (9) and expressed as µmol (g root fresh weight)$^{-1}$.

Statistical analysis: The data were analyzed using the analysis of variance (ANOVA). Significant differences between means were identified by Duncan's multiple range test. $P \leq 0.05$ was adopted as the minimum criterion of significance. Statistical analyses were performed using the Statistica™ software package.

RESULTS AND DISCUSSION

Identification of low molecular weight organic acids in *B. ruziziensis*

Figure 1 shows a typical HPLC chromatogram of standards (upper graph) or *B. ruziziensis* aqueous fraction (lower graph). The levels of organic acids in the aqueous fraction are shown in Table 1. Among the assayed acids, succinic acid and citric acid were present in higher concentrations, followed by *trans*-aconitic acid. L-Lactic acid, 2-oxoglutaric acid and fumaric acid were at lower concentrations.

Table 1. Quantification of selected aliphatic organic acids from the aqueous fraction of *B. ruziziensis*. The values were calculated from HPLC traces as shown in Figure 1.

Organic acid	Concentration ($\mu mol\ g^{-1}$)
Trans-aconitic acid	0.692 ± 0.054
Citric acid	1.741 ± 0.032
Fumaric acid	0.037 ± 0.0016
Lactic acid	0.204 ± 0.059
2-Oxoglutaric acid	0.127 ± 0.0034
Succinic acid	1.377 ± 0.179

Values are means ± SE (n = 4).

These low molecular organic acids except the *trans*-aconitic acid, are intermediates in plant primary metabolism. *Trans*-aconitic acid is an isomer of *cis*-aconitic acid and the latter is substrate of aconitase enzyme in the citric acid cycle. The *trans*-aconitic acid may be an allelopathic agent (44,46), hence, it was assayed on *I. triloba*.

Preliminary assays revealed that at concentrations lower than 200 ppm, pure *trans*-aconitic acid had no effect on seed germination of *I. triloba*. The concentration of *trans*-aconitic acid present in the aqueous fraction is very low (0.118 g *trans*-aconitic acid per g dry weight of aqueous fraction). Therefore, the concentrations of *trans*-aconitic in the aqueous fraction of *B. ruziziensis* at 1000 ppm, for example, is equal to 0.118 ppm. Despite this, we investigated the actions of *trans*-aconitic acid at higher concentrations, because it is also present at much higher concentrations in some plant species. In *B. ruziziensis*, 0.2% *trans*-aconitic acid (w/w, dry weight) was measured, whereas concentrations of 1 - 2.5% are present in mixed grasses, 3.5% in *Hordeum leporinum* L., 4.2% in *Phalaris tuberose* and 12.2% in *Delphinium hesperium* (10). High concentrations of *trans*-aconitic acid are also present in sugar cane (2% in dried sugar cane juice).

Germination and growth of *I. triloba*.

I. triloba seeds were incubated in the absence or presence of aqueous fraction of *B. ruziziensis* or *trans*-aconitic acid at concentrations ranging from 250 to 2000 ppm. To measure the seed germination indices, the germinated seeds were counted 12, 24, 36 and 48 h after incubation. The seeds of *I. triloba* started to germinate 12 h after imbibition, achieving maximum germination (76%) at 24 h (data not shown). Table 2 shows that the mean germination time of *I. triloba* remained unchanged, but the speeds of both germination and accumulated germination were reduced by both aqueous fraction and *trans*-aconitic acid. *Trans*-aconitic acid was less active, but the aqueous fraction reduced

the germination indices at 250, 1000 and 2000 ppm. *Trans*-aconitic acid caused significant effects at 2000 ppm concentration.

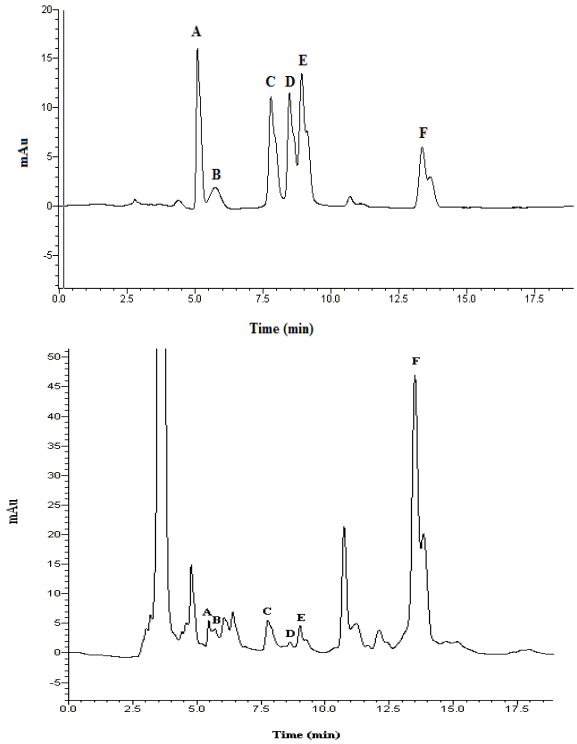

Figure 1. Upper graph: HPLC trace of 10 µL injection of standard organic acids. A: L-Lactate (900 nmol), B: 2-oxoglutarate (20 nmol) , C : citrate (300 nmol) , D : succinate (900 nmol), E : fumarate (10 nmol), F : *trans*-aconitate (10 nmol). Lower graph: HPLC trace of 10 µL injection of a solution of the aqueous fraction of *B. ruziziensis* (100 $mg.mL^{-1}$). PDA detection at 210 nm.

The growth of primary roots and hypocotyls from germinated seeds steadily increased until 48 h after incubation **(**Fig. 2). Between 12 to 48 h, the lengths of primary roots and hypocotyls increased 5.0-folds and 20-fold, respectively. The fresh and dry weights of primary roots and hypocotyls also steadily increased.

Both aqueous fraction and *trans*-aconitic acid caused changes in the growth of seedlings, which were more evident at 36 h and 48 h of incubation. At 36 h, the aqueous fraction and *trans*-aconitic acid caused a dose-dependent reduction of primary root growth.

Table 2. Mean germination time, speed of germination and speed of accumulated germination of *I. triloba* incubated for 12, 24, 36 or 48 h in distilled water (control), aqueous fraction of *B. ruziziensis* or *trans*-aconitic acid

Treatment	Conc (ppm)	Germination time ($\bar{t}$) (h)	Germination speed (S)[#]	Cumulative Germination speed (CS)
Control	0	17.35 ± 0.29	0.71 ± 0.01	3.15 ± 0.05
Aqueous fraction	250	16.89 ± 0.13	0.60 ± 0.03*	2.81 ± 0.08*
(n = 5	500	17.42 ± 0.41	0.72 ± 0.03	3.10 ± 0.09
	1000	17.35 ± 0.52	0.60 ± 0.02*	2.78 ± 0.07*
	2000	16.71 ± 0.39	0.61 ± 0.05*	2.67 ± 0.08*
Trans-aconitic acid	250	17.04 ± 0.29	0.70 ± 0.02	3.12 ± 0.07
(n = 5)	500	16.85 ± 0.21	0.71 ± 0.01	3.04 ± 0.05
	1000	17.02 ± 0.53	0.69 ± 0.05	3.05 ± 0.14
	2000	18.33 ± 0.50	0.39 ± 0.03[§]	2.14 ± 0.07[§]

[#]Seeds germinated per hour, Values are expressed as the mean ± SE. Significant differences between means were identified by ANOVA using Duncan's multiple range test ($P < 0.05$). (*) Values of seeds treated with the aqueous fraction of *B. ruziziensis* differing statistically from the values of untreated seeds (control); ([§]) Values of seeds treated with *trans*-aconitic acid differing statistically from the values of untreated seeds (control).

After 48 h growth, the length of primary roots of seedlings treated with 250 and 500 ppm of aqueous fraction reached lengths similar to controls. The aqueous fraction (1000 and 2000 ppm) reduced the length (36%) of primary roots, whereas, *trans*-aconitic acid caused 27% reduction at 250 ppm to 44% at 2000 ppm.

The effects of aqueous fraction and *trans*-aconitic acid on hypocotyl growth were different than on primary roots. Significant changes that varied with concentration were observed only 48 h after incubation. Notable, increase in hypocotyl growth was observed with aqueous fraction at 250 ppm (+ 28%) and 1000 ppm (+ 38%). However, inhibition (-28%) in hypocotyl growth occurred at 2000 ppm concentration. The *trans*-aconitic acid concentrations of 250 ppm (+26%) and 2000 ppm (+21%) increased the hypocotyl growth.

The total fresh weights of primary roots and hypocotyls of seedlings grown with aqueous fraction varied from inhibition at 36 h after incubation, to an increase at 48 h, showing the opposite effects on the growth of primary roots and hypocotyls. The corresponding values of dry weights were significantly different from their respective controls at 48 h after incubation. The dry weights of primary roots and hypocotyls were increased by 22% (250 ppm), 12% (500 ppm) and 22% (1000 ppm), but there was reduction of 14% (2000 ppm).

The effects of *trans*-aconitic acid on fresh and dry weights of seedlings were less pronounced. There were significant changes only with *trans*-aconitic acid at 2000 ppm. There was reduction in both the fresh and dry weights of seedlings at 36 h after incubation, however fresh weight increased after 48 h.

Voll *et al.* (45) observed higher inhibition of growth from the *trans*-aconitic acid than our results. They tested the effects of 2.5 mM *trans*-aconitic acid (435.25 ppm) on germination and growth of *I. triloba* from different locations of Paraná State (Brazil) and observed 73% and 94% inhibition in growth of stems and roots, respectively. The

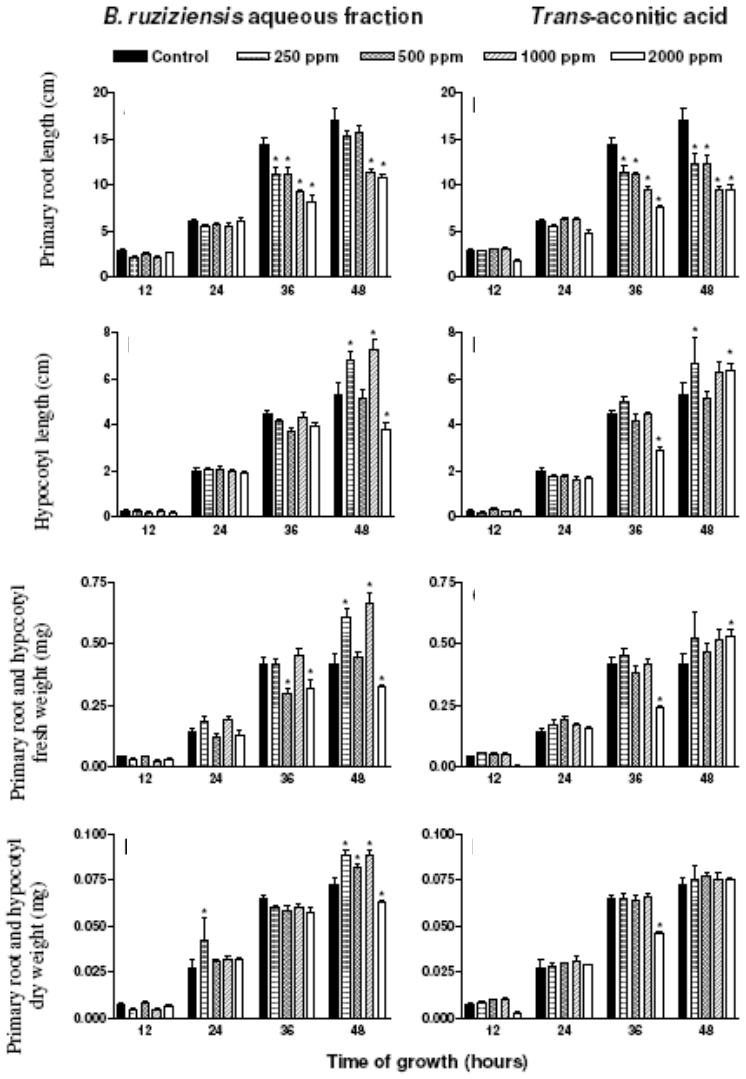

Figure 2. Effects of aqueous fraction of *B. ruziziensis* and *trans*-aconitic acid on primary root length, hypocotyl length, primary root and hypocotyl fresh weights and primary root and hypocotyl dry weights of *I. triloba* incubated for 12, 24, 36 or 48 h. The values are the means ± SE (n = 5). *Significant differences between seeds treated with the aqueous fraction of *B. ruziziensis* or trans-aconitic acid by ANOVA using Duncan's multiple range test. The respective controls are indicated ($P < 0.05$).

experimental conditions of Voll *et al.* (45) were very different than conditions used in this study. We incubated the seeds in germination paper for 2 days, whereas Voll *et al.* (45) incubated the seeds in an agar medium for 10-12 days.

Respiration in *I. triloba* primary roots

The respiratory activity of *I. triloba* primary roots was measured in the seedlings treated for 36 h with aqueous fraction or *trans*-aconitic acid at 250 to 2000 ppm concentrations (Fig. 3). Inhibition of cytochrome oxidase with potassium cyanide (KCN), showed major role of KCN-tolerant respiration to the total respiration in primary roots at 36 h after growth. It was 40% and 33%, respectively, in Panels A and B of Figure 3.

The total respiration rate of root apices was inhibited (26%) in *B. ruziziensis* aqueous fraction at 250 ppm. However, at higher concentrations total respiration was similar to control. This increase resulted from the opposing effects of aqueous fraction on KCN-sensitive and KCN-tolerant respiration. KCN-sensitive respiration was inhibited, but contrarily the KCN-insensitive respiration was increased. At 2000 ppm of the aqueous fraction the KCN-sensitive respiration was reduced from 60% in control to 39%, while the KCN-tolerant respiration increased from 40% (control) to 61%.

A similar trend was observed in presence of *trans*-aconitic acid (Fig. 3 Panel B). At 2000 ppm, the KCN-sensitive respiration was reduced from 67.4% in control to 37%, while the KCN-insensitive respiration increased from 33% (control) to 63%.

The simplest explanation of how the reduction in growth of primary root is associated with reduction in KCN-sensitive respiration [cytochrome-oxidase (COX) pathways respiration] is either due to the inhibition of COX pathways of mitochondrial electron transport, the citric acid cycle or due to the supply of substrate to citric acid cycle.

Trans-aconitate is competitive inhibitor of aconitase (36). It was verified that *trans*-aconitate causes accumulation of citrate and decreases the respiratory rates of oxygen consumption in rat kidney slices.

Respiration in mitochondria isolated from *I. triloba* primary roots.

The hypothesis of inhibitory effects of aqueous fraction or *trans*-aconitic acid on aconitase was tested in the isolated mitochondria by measuring the oxygen consumption due to citrate oxidation. For this experiment, mitochondria were isolated from the primary roots of seedlings grown for 72 h, because there was not enough material for mitochondria isolation in earlier stages of seedling growth. The oxygen consumption due to citrate oxidation was not reduced, either by *trans*-aconitic acid or by the aqueous fraction (Fig. 4). Contrarily the aqueous fraction at 500 ppm concentration increased the respiration. *Trans*-aconitic acid also increased the citrate oxidation at 500 and 1000 ppm. These results clearly indicated that *trans*-aconitic acid and the components of aqueous fraction did not act as aconitase inhibitors in *I. triloba* seedlings. Additionally, they did not act as inhibitors of respiratory chain components. Therefore, we speculate that the inhibition of KCN-sensitive respiration of primary roots was due to interference in any part of energy metabolism pathways that precedes the first reaction of citric acid cycle. Additionally, a time-dependent effect on citric acid or respiratory chain components cannot be excluded because the respiration of intact tissues was measured 36 h after exposure to aqueous fraction and *trans*-aconitic acid (Figure 3).

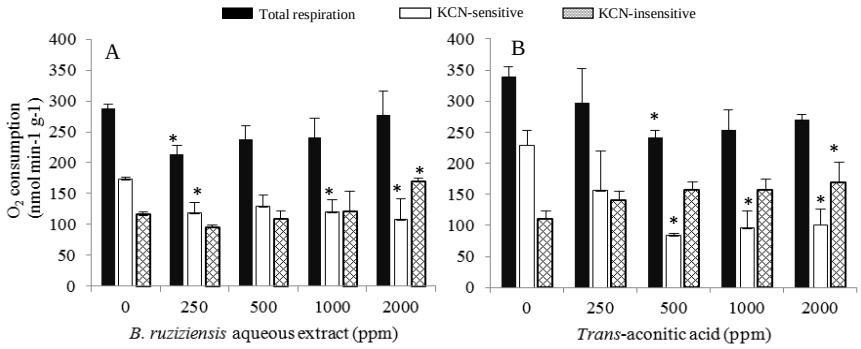

Figure 3. Effects of aqueous fraction of *B. ruziziensis* (A) and *trans*-aconitic acid (B) on the respiratory activity of *I. triloba* primary roots grown for 36 h. Root tip samples were removed from seedlings and immediately added to the oxygen electrode vessel, containing 2.0 mL of nutrient medium in the absence or presence of 270 µM KCN. Oxygen consumption was followed polarographically over approximately 12-15 min. Total respiration – rate of oxygen consumption in the absence of inhibitors; KCN-insensitive respiration – rates of oxygen consumption in the absence of KCN; KCN-sensitive respiration – difference between total respiration and KCN-insensitive respiration. The values are the means ± SE (n = 4). *Significant differences between seedlings treated with the aqueous fraction of *B. ruziziensis* or *trans*-aconitic acid and the respective controls were identified by ANOVA using Duncan's multiple range test ($P < 0.05$).

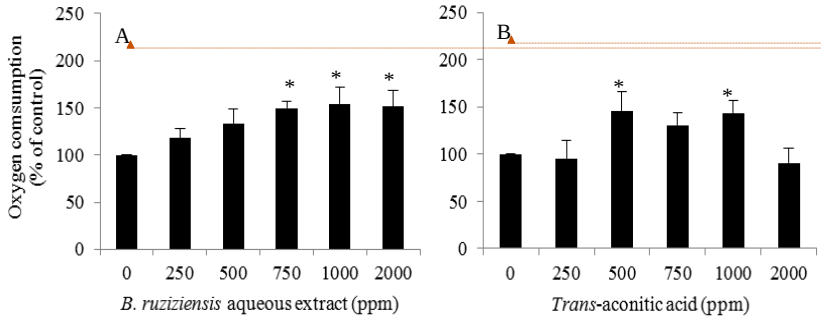

Figure 4. Effects of aqueous fraction of *B. ruziziensis* (A) and *trans*-aconitic acid (B) on oxygen consumption due to citrate oxidation in mitochondria isolated from the primary roots of *I. triloba* grown for 72 h. Mitochondria (0.26-0.48 mg protein) were added to the oxygen electrode vessel containing 2.0 mL of reaction medium, and the reaction was initiated by the addition of 10 mM citrate. Oxygen uptake was followed polarographically for approximately 5 min. Values are the means ± SEM of 4 independent mitochondrial preparations. Significant differences between the values the treatment and the respective controls were identified by ANOVA using Duncan'stest, (*): $P<0.05$).

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Activity of lipoxygenase, MDA and conjugated diene in *I. triloba* primary roots.

The KCN-tolerant respiration increased with both *trans*-aconitic acid and aqueous fraction probably due to oxidative stress. KCN-tolerant respiration in intact tissues accounts for oxygen that is converted into superoxide and H_2O_2 (35) or used by mitochondrial alternative oxidase (AOX) and other cytosolic or membrane-bound oxidases, including lipoxygenases. All these processes increases under the oxidative stress (7,33,34,41). Indeed the increased activity of AOX was supported by the increased respiratory activity of isolated mitochondria (Fig. 4). The induction of oxidative stress in seedlings was determined by assessing the lipoxygenase activity, MDA and conjugated diene content (Tables 3 and 4). The aqueous fraction (Table 3) or *trans*-aconitic acid (Table 4) did not change the activity of lipoxygenase. However, both aqueous fraction and *trans*-aconitic acid increased the MDA and conjugated diene content. This increase was significant at 2000 ppm concentration, which increased the KCN-tolerant respiration.

Table 3. The effects of the aqueous fraction of *B. ruziziensis* on lipoxygenase activity and on MDA and conjugated diene content in roots from *I. triloba* seedlings grown for 36 h

Aqueous fraction of *B. ruziziensis* (ppm)	Lipoxygenase activity (nmol min^{-1} mg $protein^{-1}$) (n = 7)	MDA [µmol (g fresh $weight)^{-1}$] (n = 5)	Conjugated dienes [µmol (g fresh $weight)^{-1}$] (n = 5)
0	906.0 ± 59	16.83 ± 1.67	2203.78 ± 197.16
250	886.0 ± 90	15.92 ± 2.30	2107.91 ± 196.40
500	954.0 ± 54	18.79 ± 2.02	2168.26 ± 146.23
1000	758.5 ± 75	19.24 ± 2.67	2199.62 ± 145.50
2000	896.2 ± 86	23.62 ± 4.13*	2515.27 ± 121.39*

Values are expressed as the mean ± SE. Significant differences between means were identified by ANOVA (repeated measures) using Duncan's multiple range test ($P < 0.05$). (*) Values of seedlings treated with the aqueous fraction of *B. ruziziensis* differing statistically from the values of untreated seedlings (control).

Table 4. The effects of *trans*-aconitic acid on lipoxygenase activity and on MDA and conjugated diene content in roots from *I. triloba* seedlings grown for 36 h

Trans-aconitic acid (ppm)	Lipoxygenase activity (nmol min^{-1} mg $protein^{-1}$) (n = 7)	MDA [µmol (g fresh $weight)^{-1}$] (n = 5)	Conjugated dienes [µmol (g fresh $weight)^{-1}$] (n = 5)
0	975.9 ± 113	14.67 ± 1.31	2310.97 ± 105.31
250	1219.0 ± 117	14.87 ± 1.49	2081.29 ± 114.03
500	1059.0 ± 73.57	19.41 ± 2.50*	2507.79 ± 99.73
1000	1333.8 ± 196	17.54 ± 1.04	2437.74 ± 106.74
2000	1303.3 ± 65	20.31 ± 1.84*	2565.71 ± 24.42*

Values are expressed as the mean ± SE. Significant differences between means were identified by ANOVA (repeated measures) using Duncan's multiple range test ($P < 0.05$). (*) Values of seedlings treated with trans-aconitic acid differing statistically from the values of untreated seedlings (control).

The KCN-sensitive respiration and primary root growth were inhibited at lower concentrations (Figure 3), suggesting that the impaired respiratory activity related to energy metabolism is stronger than the effects in inducing oxidative stress.

The aqueous fraction of *B. ruziziensis* possesses compounds that are phytotoxic to *I. triloba*. Although *trans*-aconitic acid exerted effects similar to aqueous fraction, it is not the main compound responsible for the effects of aqueous fraction in *I. triloba*, because its content is very little in this fraction. Voll *et al.* (43) also reported a similar phenomenon. They verified that the *B. plantaginea* extract containing known concentrations of aconitic acid (43.5 to 174 ppm) was more inhibitory to *Acanthospermum hispidum* L. and *C. benghalensis* than pure aconitic acid. The *trans*-aconitic acid may contribute to the phytotoxicity of other plants that produce *trans*-aconitic acid in quantities more than that produced by *B. ruziziensis* or *B. plantaginea* (10).

The aqueous fraction of *B. ruziziensis* contains unknown phytotoxic compounds which may be allelopathic (13,31). The amount of *Brachiaria* straw used in field studies varies from 3 t - 6 $t.ha^{-1}$ (2,27,31). In our study, 18.4 g aqueous fraction was obtained from 1 kg dry *B. ruziziensis* leaves and stems. In 6 t straw, approximately 110.4 kg aqueous fraction could be theoretically released into the soil. This concentration is equal to 1.10×10^5 ppb (110 ppm) in soil on weight basis distributed in 10 cm depth (26). It is difficult to predict the concentration of active compounds in aqueous fraction of soil solutions because several factors (pH, temperature, the nature of the soil, cation and anion exchange reactions and microorganisms) can influence the availability of organic compounds for the growth of receptor plants (8). The aqueous fraction inhibited the growth of *I. triloba* roots at concentrations of 250 ppm and higher (water solutions). The concentration of compounds in soil water would then be the same or higher than the active range of concentrations revealed in our study with *I. triloba*. It was concluded that *B. ruziziensis* residues might release compounds with allelopathic activity in *I. triloba*, a property that needs more research in field conditions.

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