South African Journal of Botany 93 (2014) 167–178

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Physiological and biochemical mechanisms of allelochemicals in aqueous extracts of diploid and mixoploid *Trigonella foenum-graecum* L.

Faten Omezzine [a,*], Afef Ladhari [a], Rabiaa Haouala [b]

[a] Department of Biology, Faculty of Sciences of Bizerte, University of Carthage, Amilcar, 1054, Tunisia, (UR13AGR05)
[b] Department of Biological Sciences and Plant Protection, Higher Agronomic Institute of Chott-Mariem, University of Sousse, Chott-Mariem, 4042, Sousse, Tunisia, (UR13AGR05)

ARTICLE INFO

Article history:
Received 10 March 2014
Received in revised form 10 April 2014
Accepted 16 April 2014
Available online xxxx

Edited by J Van Staden

Keywords:
Fenugreek
Ploidy level
Developmental stage
Mode of action
Aqueous extract
Lettuce

ABSTRACT

This study was conducted to evaluate the effect of shoot aqueous extracts of diploid and mixoploid fenugreek at vegetative, flowering and fruiting stages on some physiological and biochemical processes in lettuce. The allelochemicals stress was registered as the result of aqueous extract application, which was added to the Hoagland nutrient solution at concentration corresponding to IC_{50} (50% inhibition of germination or root growth). The germination inhibition seems to be correlated with membrane deterioration as proved by a strong electrolyte leakage, increase in malondialdehyde (MDA) content, and mitochondrial respiration disruption due to a decrease in dehydrogenases activity. These disruptions were recorded with all test extracts, especially fruiting stage extract of diploid and mixoploid plants. For seedling growth inhibition, the roots showed the same interference, especially in the presence of aqueous extract of plant material harvested at the vegetative stage for diploid and at flowering of mixoploid plants. Chlorophyll content was slightly reduced while carotenoid content was significantly reduced. The lettuce seedlings have circumvented the allelochemicals stress, by *i*) increasing the phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activity, *ii*) accumulation of proline and *iii*) the production of secondary metabolites with antioxidant potent, such as polyphenols, flavonoids and alkaloids. The importance of these phenomenons varied with the extract origin and target organ, which is in favor of speculating on the allelochemicals specificity and on the change in the chemical composition of different extracts. Also, understanding of the different mechanisms of allelochemicals may provide a basis for the development of growth regulators and natural pesticides to boost up production in sustainable agriculture.

1. Introduction

Environmental stress factors limit the agricultural productivity and many of these factors are related to the metabolic processes. The plant response to stress depends on the duration, severity and rate of imposed stress (Munne-Bosch and Alegere, 2004). Under natural conditions, multiple stresses develop progressively and gradually, eliciting morphological, physiological and biochemical responses. Plants produce various secondary metabolites some of them are known to be allelochemicals, whose action can be beneficial or detrimental to the growth and development of the receptor species. In the latter case, the effect is described by a biotic stress "allelochemical stress" (Pedrol et al., 2006). Indeed, it is reported that allelochemicals reduce cell division (Sánchez-Moreiras et al., 2008) directly by affecting many physiological and biochemical reactions (Einhellig, 2002). Therefore, they influence on the growth and development of plants (Lara-Núñez et al., 2009). According to Einhellig (1986, 1995) and Macias et al. (2001), specifically to a given allelopathic compound mode of action has not yet been studied and a lot of additional information is required. One of the most important limitations that reduced attempts to learn about how allelochemicals affect the growth of the receiving plant is the lack of sufficient of composed quantities for to study the effects on physiological processes and cellular mechanisms (Einhellig, 1995). In addition, difficulties also limit these studies that come from the multitude of potential molecular targets (Einhellig, 1986). Thus, allelochemicals have several molecular targets and are known to effect many cellular processes in target plants, viz. stomatal closure (Barkosky et al., 2000), cell division (Anaya and Pelayo-Benavides, 1997), membrane permeability (Galindo et al., 1999), absorption of nutrients (Baar et al., 1994), photosynthesis (Baziramakenga et al., 1994), respiration (Abrahim et al., 2000), transpiration, efficiency of photosystem II (PSII), the synthesis of ATP, the phytohormone metabolism, the production of reactive oxygen species, gene expression and other metabolic processes (Blum, 2005).

Previous studies (Omezzine and Haouala, 2013; Omezzine et al., 2014) have shown that different extracts of the aerial parts of diploid and mixoploid *T. foenum-graecum*, were toxic for lettuce germination and growth. The phytotoxicity degree was largely dependent on the

* Corresponding author at: Higher Agronomic Institute of Chott-Mariem, BP 47, 4042 Chott-Mariem, Sousse, Tunisia. Tel.: +216 97080195; fax: +216 73327591.
E-mail address: faten.omez@yahoo.fr (F. Omezzine).

http://dx.doi.org/10.1016/j.sajb.2014.04.009
0254-6299/

developmental stage at which the material was collected, but also with the ploidy level. In this study, the aqueous extracts of *Trigonella foenum-graecum* (diploid and mixoploid) were used as a factor of biotic stress on lettuce and our aim is to compare their effects on a number of biochemical and physiological parameters in lettuce to understand their mechanisms of germination and growth inhibition. The parameters evaluated are: the secondary metabolites production, lyase enzyme activity, cell metabolic activity, content of photosynthetic pigments and membrane integrity assessed by measuring the electrolyte leakage and the lipid peroxidation.

2. Material and methods

2.1. Plant material and mixoploidy induction

The mixoploid plants of *T. foenum-graecum* were obtained following seed treatment with 0.05% colchicine solution, according to Omezzine et al. (2012). Fenugreek treated and untreated seeds were sown in field under natural conditions in March 2011. The mixoploidy confirmation was done by flow cytometry and stomata and pollen grain size (Omezzine et al., 2012). Aerial parts of diploid (plant from untreated seeds) and mixoploid (plant from colchicine treated seeds) plants were harvested at vegetative (plants with 8 leaves), flowering (50% of flowers are blossomed) and fruiting stages (50% of the pods have reached a typical length). Fresh plants were washed out under tap water, then oven-dried at 60 °C for 72 h, powdered and used for extraction.

2.2. Lettuce growth and treatment conditions

Seeds were germinated in Petri dishes at room temperature in the dark. Seven-day old seedlings were irrigated with distilled water during the first week. Uniform seedlings were subsequently cultured individually in a hydroponic system containing a complete Hoagland's medium (Hoagland and Arnon, 1950) diluted eightfold in a greenhouse (16 h light/8 h dark at 20/17 °C). After two weeks, the plants were divided into batches cultured in the absence (control group) and in the presence of aqueous extracts of diploid and mixoploid fenugreek plants, harvested at the vegetative, flowering and fruiting stage, and prepared at a concentration inducing a reduction of 50% root growth (IC_{50}). The culture media were aerated continuously and the renewal was done every 2 days. At the end of the treatment period (7 days), the plants were harvested and separated into leaves and roots.

2.3. Electrolyte leakage

The electrolyte leakage (EL) was determined as described by Lutts et al. (1996). Seeds or roots of fresh lettuce seedlings were cut and placed in test tubes containing 15 mL of distilled water for controls and 15 mL of each aqueous extract of diploid and mixoploid fenugreek plants for treatments. The tubes were incubated at room temperature for 24 h and 48 h and the initial electrical conductivity of the medium (EC_1) was measured using a digital conductivity meter (type BCT-4308). The samples were autoclaved at 121 °C for 20 min to release all electrolytes, cooled down to 25 °C and the final electrical conductivity (EC_2) measured.

The electrolyte leakage (EL) was calculated according to the following formula (Lutts et al., 1996):

$$EL = (EC_1/EC_2) \times 100.$$

2.4. Lipid peroxydation

Frozen samples (200 mg) (root and leaves) were homogenized with a mortar kept on ice and thoroughly mixed with 2.5 mL of 67 mM phosphate buffer (pH = 7) and 0.05 g PVP, which adsorbs polyphenols. After centrifugation (2000 g for 15 min at 4 °C), the supernatant was used to determine lipid peroxidation (Doblinski et al., 2003). A 750 μL of enzyme extract was added to 3 mL of 0.5% TBA (prepared in 20% TCA). The homogenate was incubated at 90 °C for 10 min. The reaction was stopped quickly by cooling the mixture in ice. Then, the mixture was centrifuged and the supernatant absorbance was measured at 532 and 600 nm, and the MDA concentration was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Doblinski et al., 2003).

2.5. Cell metabolic activity

The fresh plant material (100 mg) of germinated seeds or seedlings of lettuce grown in the absence or the presence of fenugreek extracts was washed and dried quickly between blotting paper, then incubated in 5 mL of TTC (0.2%, pH = 7) at 37 °C for 4 h in the dark. The reaction was stopped by adding 0.5 mL of sulfuric acid (1 M). Thereafter, the plant material was removed, washed with distilled water, dried quickly between filter paper and ground in a mortar placed in ice containing 3.5 mL of ethyl acetate. The homogenate was filtered through a paper Whattman No. 1 and the volume was adjusted to 7 mL with ethyl acetate. The absorbance was measured at 485 nm and the amount of formazan was calculated as follows (Sampietro et al., 2006):

$$\text{Formazan content}(\%) = DO_{485}\ \text{treatment}/DO_{485}\ \text{control}.$$

2.6. Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia lyase (TAL) activities

Extraction and assay of enzymes were prepared under optimal conditions of pH and temperature. The fresh plant material (1 g) was ground in a mortar placed in ice (5 °C) containing 20 mL of borate buffer (0.1 M, pH = 8.7). The homogenate was filtered through filter paper Whattman No. 1. After centrifugation at 15,000 tr/min at 5 °C for 10 min, the supernatant recovered constituted the crude enzyme extract.

The PAL activity was determined according to Takayoshi and Kawamura (1964). The initial optical density (DO_1) of the reaction mixture (1 mL) containing 50 mM L-phenylalanine and 0.2 mL of the crude enzyme extract was determined at 270 nm. After incubation at 40 °C for 90 min, the reaction was stopped by placing the tubes in ice, and the staining intensity was determined at 270 nm (DO_2).

For the TAL, the fresh plant material (1 g) was ground in a mortar placed in ice (5 °C) containing 20 mL of borate buffer (0.1 M, pH = 9). The homogenate was filtered through filter paper Whattman No. 1. After centrifugation at 15,000 tr/min at 5 °C for 10 min, the supernatant recovered constituted the crude enzyme extract.

The TAL activity was performed according to Takayoshi and Kawamura (1964). The initial optical density (DO_1) of the reaction mixture (1 mL) containing 10 mM L-tyrosine and 0.3 mL of crude enzyme extract was determined at 333 nm. After incubation at 40 °C for 90 min, the reaction was stopped by placing the tubes in ice and staining intensity was determined at 333 nm (DO_2).

2.7. Proline content

Proline in lettuce roots and leaves was extracted and analyzed according to Bates et al. (1973). Ten milligram (10 mg) of dry plant material was mixed with 1.5 mL aqueous sulfosalicylic acid (3%, w/v). The homogenate was centrifuged at 13,000 tr/min for 10 min, and the supernatant was transferred to a fresh 1.5 mL tube. The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic and 20 mL 6 M H_3PO_4) and incubated at 100 °C for 1 h. The reaction was terminated by placing the tube in an ice bath. The reaction mixture was vigorously