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Acclimatization of *Pisum sativum* L., grown in soil contaminated with veterinary antibiotics, an attribute of dose hormetic response of root metabolites

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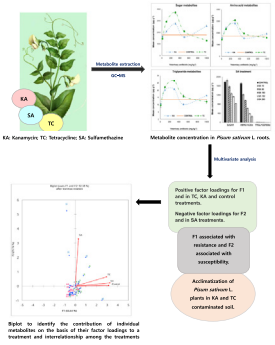
HIGHLIGHTS

- Dose-dependent hormetic effect of KA and TC on root metabolite concentration.
- 'Defense-related metabolites up-regulated in abundance in comparison with control.
- Control, TC, and KA had positive factor scores for F1 and a negative factor score for F2, which was just the opposite for SA.
- Higher positive factor scores of F1 associated with resistant clusters of KA and TC, and higher negative factor scores of F2 associated with SA susceptibility.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant-veterinary antibiotic interaction has been widely studied, however, to the best of our knowledge acclimatization studies with regard to changes in plant root metabolites has not been reported so far. The purpose of this study was to examine the changes in the metabolome of pea roots under antibiotic stress and their role in acclimatization. *Pisum sativum* L. was grown in soil contaminated with three commonly used veterinary antibiotics — kanamycin (KA), sulfamethazine (SA), and tetracycline (TC). In response to antibiotic stress, plants accumulated different types of low molecular weight compounds that provided protection from stress by contributing to ROS detoxification, protection of membrane integrity, efficient signaling, cell wall function, and cellular osmotic adjustment (glucose, galactose, myo-inositol, stigmasterol, octadecadienoic acid, L-proline). The concentration of amino acid, sugar, and triglyceride metabolites in KA and TC samples showed a dose-dependent biphasic (hormesis) fluctuation. This was mirrored in the metabolite abundance as well as the physiological attributes (mycorrhizal colonization, GST function, nutrient assimilation), which helped in the acclimatization without the loss of normal plant function. SA, on the other hand, had progressive toxic effects with increasing concentration. PCA revealed the differences to be due to SA treatments and in sterol and terpenoid metabolites.

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1. Introduction

In recent years, *veterinary antibiotics* (VA's) have frequently been reported in the environment. Animals are unable to metabolize the

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administered therapeutic and non-therapeutic VA's completely. The undigested VA's, excreted out as either the parent compound or their breakdown metabolites, pose a threat to the natural environment. Changes in the natural growing environment of plants present considerable challenges regarding growth, competition with neighboring plants and also their response to biotic and abiotic stress (Walters, 2004). Acclimatization/phenotypic plasticity enables plants to withstand environmental dynamism, for both short and long timescales and is governed by genes that determine not only the character of an organism but also the degree of responsiveness of that character to environmental stimuli (Bradshaw, 2006). Acclimatization is measured by the plant's ability to change the way they grow (morphological traits) and function (metabolic traits). Changes in plant metabolites are at the heart of plant developmental processes, underpinning many ways in which plant respond to the environment.

Plant metabolites are vital not only for plants themselves but also aid in their interactions with the environment. Additionally, secondary metabolites play crucial roles in providing defenses against biotic and abiotic stresses. Metabolites are the heart of a plant's developmental process and are sensitive to abnormal changes in the growing environment. The immense quantitative, as well as qualitative diversities in metabolites, make plants the perfect models to scrutinize the biosynthetic pathways and regulation of metabolites. The purpose of this study was to examine the acclimatization of *Pisum sativum* L. plants under VA's stress with regards to the changes in the metabolome of pea roots. Plant roots are imperative to the healthy growth and development of plants. They not only provide nutrient but are also involved in maintaining complex biochemical and enzymatic soil-plant interactions; therefore, being the first plant organ to come in contact with soil and its constituents. Also, the non-targeted profiling of metabolites in biological samples is at present considered possible complementation to protein and transcript profiling technologies. Plants were grown in soil contaminated with *kanamycin* (KA), *sulfamethazine*, and *tetracycline* (TC) for 49 days. Both polar and non-polar root metabolites were profiled along with some secondary experiments for root tip structure, mycorrhizal colonization, antioxidant function, electrolyte leakage and nutrient assimilation.

A dose-dependent hormetic effect of KA and TC antibiotics was evident from the study. SA antibiotics, on the other hand, had an adverse effect. A higher abundance of plant metabolites required for signaling (myo-inositol), membrane integrity (proline), cell wall structure (glucose and galactose derivatives) and antioxidant function (octadecadienoic acid) were recorded in VA's treated samples in comparison to control. The roots of *Pisum sativum* L. plants, therefore, respond to antibiotic stress by efficient signaling increasing their defensive and antioxidative function. The changes incurred by the root metabolites upon VA's exposure resulted in either the acclimatization of pea plant physiology to KA and TC antibiotics or as toxicity to SA antibiotics.

2. Materials and methods

Leguminous plant *Pisum sativum* L. (garden pea), family Fabaceae was grown in an open greenhouse for 49 days. The soil used in this study was sandy loam soil (pH: 5.57, total OM: 0.5%, total N, P, K: 518, 379.1, 3400 mg kg^{-1}, respectively) obtained from an agriculture field in Chonbuk National University was used. Livestock manure mixture (pH: 6.4, total OM: 53.22%) was purchased from an online vendor. Inorganic fertilizer was added at the rate of 1.5 kg per 50 kg soil to obtain final pH: 5.59, total N, P, and K: 3257.8, 455.6 and 3647 mg kg^{-1}. VA's namely kanamycin (KA), sulfamethazine (SA) and, tetracycline (TC) were selected owing to their differences in molecular weight, half-life, and behavior in soil (Table S1). Plant height, root length and leaf number after 15 days of growth in a range of VA's concentrations (0–1000 mg kg^{-1}) was the measure of selecting the final concentration of VA's used in the study. For SA concentrations above 200 mg kg^{-1}, no visible growth was observed. On the other hand, pea plants were able to grow even at 1000 mg kg^{-1} KA and TC concentration. The final five concentrations consisted of both environment relevant (10 mg kg^{-1}) as well as concentrations not likely to be found in the environment (SA: 50, 100, 150, 200 mg kg^{-1}; KA, TC: 100, 300, 500, 1000 mg kg^{-1}). The rationale behind the selection of such high dosages being the working concentration to include both the extremes and the between. To eliminate the influence of soil OM concentration on the behavior of VA's, the total OM of soil was made to 2.55% by the addition of manure mixture (20 g manure per 200 g soil). VA's were firstly by mixed with livestock manure and then incorporated into the soil. Four pea seeds (pre-soaked in lukewarm water) per pot (12 cm × 12 cm) was planted at a depth of 1 cm, watered (80% moisture content), weighed and placed in a greenhouse. Plants were watered 2–3 times a week with regular tap water. In a separate study, the IC_{50} (inhibiting concentration) values for percentage root growth and germination index for KA, SA, and TC were found to be — 334.4, 245.3, and 311.6 mg kg^{-1} and 292.5, 121.3, and 247.6 mg kg^{-1}, respectively.

2.1. Metabolite extraction

The reagents used were of analytical grade and purchased from Sigma Aldrich Korea. The method of Lytovchenko et al., 2009 was used. Briefly, 0.1 g of homogenized root sample was extracted in l.5 ml MeOH and incubated at 70 °C for 15 min with constant agitation. Following centrifugation (14,000*g*, 5 min) the supernatant was transferred to a glass vial, and the pellet re-extracted with chloroform and incubated at 35 °C with constant agitation. Supernatants were pooled together, and H_2O (1 ml) and CH_3Cl (1 ml) added and centrifuged after vigorous shaking. 0.5 ml aliquots of the CH_3Cl were dried with rotavapor and concentrated with N_2 gas after the addition of 1 ml CH_3Cl. 1.2 ml aliquots of the non-polar phase dried under N_2 stream for the analysis of nonpolar metabolites.

The polar N—H and O—H groups were derivatized by resuspending in 50 μl of methoxyamine hydrochloride solution (20 mg ml^{-1} in pyridine) and incubated (2 h, 60 °C with constant agitation). Acidic protons are trimethylsilylated with 200 μl *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and incubated (60 °C, 30 min with constant agitation). For complete derivatization, samples were allowed to stand at room temperature overnight. Nonpolar metabolite extracts were also derivatized with MSTFA. After reconstituting the N_2 dried samples in 1 ml hexane, 200 μl MSTFA was added and heated for 2 h at 50 °C.

2.2. GC–MS instrumentation

Samples (polar and non-polar except for amino acids) were cooled down to room temperature and injected into QI-2010 plus GC–MS with DB-5 MS column and helium carrier gas (1 ml min^{-1}, constant). GC conditions were set as injection temp 250 °C; injector splitless 1.0 split ratio; resting oven temperature 60 °C; and interface temperature 300 °C. Following injection of 0.5 μl sample, the oven was ramped to 160 °C at the rate of 25 min^{-1}, the temperature was ramped to 240 °C at the rate of 4 min^{-1} and held for 3 min. Finally, the temperature was ramped to 290 °C and held for an additional 8 min before being cooled rapidly to 60 °C for the next run. For amino acid samples 1 μl sample was injected in split mode (10.0 split ratio) with the resting oven temperature of 100 °C. The oven temperature is ramped up to 290 °C followed by a 20 min^{-1} ramping up to 310 °C for 11 min.

Mass spectrometry analysis was conducted in positive electron ionization mode with helium gas flow into the chamber set at 1.51 ml min^{-1} for amino acids and 1.21 ml min^{-1} for all other samples. The source temperature was held at 200 °C. Detector signal for amino acids recorded from 2 to 21 min and ions scanned across 45–450 *m/z*.

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