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Re-start of olive waste vermicompost through addition of tryptophan and its effects on indole-3-acetic acid in pepper rhizosphere when used as soil amendment

Beatriz Moreno, Rogelio Nogales, Lourdes Sánchez, Emilio Benítez*

Department of Environmental Protection, Estación Experimental del Zaidín (EEZ), CSIC, Profesor Albareda 1, 18008 Granada, Spain

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ABSTRACT

In this work, we aimed to test the capacity of an exogenous source of tryptophan to stimulate the bacterial biosynthesis of the phytohormone indole-3-acetic acid (IAA) in agro-industrial by-products, trying to gain insight into the role of key genes involved in the indole-3-pyruvic acid pathway (IPA) for bacterial IAA synthesis. Sunflower-oil cake was mixed with a 9-months-old vermicompost olive wastes and then vermicomposted for 60 days using earthworms *Eisenia foetida*. Synthetic tryptophan and no tryptophan source were used as controls. For this, a novel set of primers targeting bacterial aldehyde-dehydrogenase (ALDH)-encoding genes involved in the last step of the IPA route was designed and tested, and a method involving ultrasound-assisted extraction of IAA and subsequent analysis by liquid chromatography–tandem mass spectrometry was optimized. Re-starting the vermicomposting process increased bacterial abundance and the number and the expression of ALDH genes but not the IAA content in the final vermicomposts, meaning that the transcription of the ALDH genes could be not quite high enough to change the overall level of the existent IAA. After that, the effects of the resulting vermicompost were evaluated on the bacterial microbiome of the pepper (*Capsicum annuum* L.) rhizosphere. When used as amendments, ALDH gene expression was detected in the pepper's rhizosphere after 3 months of growth only when vermicompost incorporating sunflower cake was used, where the highest levels of IAA were also detected. The results indicate the presence of the ALDH genes in the chromosome of most of vermicompost-borne bacteria, probably not specific for IAA synthesis, and that the IPA pathway may not be the main biosynthesis pathway for IAA in vermicompost-borne bacteria.

1. Introduction

Most land-based food sources are being affected by climate change so that improved plant productivity will be necessary in order to sustain increasing population growth. This includes the design of environmentally responsible crop-management systems to meet economic profitability while enhancing soil quality, i.e. "the capacity of soil to function" (Jackson-Smith, 2010). Soil organic C plays a critical role in maintaining soil functions, particularly in semi-arid areas. However, there is general deficiency of organic carbon in most of agricultural soils (Guo and Gifford 2002). Recycling of organic wastes has been identified as a smart strategy for sustainable agriculture as well as for a pollution-free environment (Despommier, 2010).

One of the most problematic solid biowastes, for which disposal has become an enormous dilemma in the Mediterranean area, are olive-mill solid wastes. The olive-oil extraction process generates huge amounts (about 4–5 × 10^9 kg per year in Spain) of olive-mill solid waste. They exhibit phytotoxic and antimicrobial effects which limit direct soil application, and thus its current disposal represents a great economic and technical problem for producers. Vermicomposting is one of the best-known processes for the biological stabilization of olive wastes (Vivas et al., 2009; Castillo-Diaz et al., 2013). Vermicomposting is a decomposition process that involves bio-oxidation and stabilization of the waste as a result of the interactions between some species of earthworms and microorganisms into a stabilized product (Dominguez, 2004). This product, called vermicompost, contains relatively high contents of humic-like compounds, nutrients, active microorganisms and enzymes that greatly enhance the soil fertility when applied to soil (Benitez et al., 2005; Fernandez-Gomez et al., 2011). Vermicompost also shows hormone-like activity that improves plant nutrition and growth. The bioactivity of vermicompost has usually been related to the molecular characteristics of the humic acids contained therein (Atiyeh et al., 2002), but emerging reports also offer evidence of plant growth-promoting rhizobacteria (PGPR) in the vermicompost which are cap-

* Corresponding author.
E-mail address: emilio.benitez@eez.csic.es (E. Benítez).

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able of producing hormone-like compounds (Puga-Freitas et al., 2012; Blouin et al., 2013). Thus, indole-3-acetic acid (IAA), a reciprocal signalling molecule in bacteria–plant interactions that modulates plant growth and development (Lambrecht et al., 2000), has been retrieved from vermicomposts (Zhang et al., 2015). Biosynthesis of IAA has been well described for bacteria and it is assumed that over 80% of the rhizosphere bacteria are capable of synthesizing IAA. Starting with tryptophan, three main pathways have been described for bacterial synthesis of IAA: the indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), and indole-3-acetonitrile (IAN) pathways, the first two being the most common routes for IAA biosynthesis (Spaepen and Vanderleyden, 2011). It is generally accepted that plant-pathogenic bacteria synthesize IAA mainly via the IAM pathway (Costacurta and Vanderleyden, 1995) while IPA is the predominant pathway in plant-beneficial bacteria (Prinsen et al., 1993; Theunis et al., 2004). In any case, the inactivation of key genes has indicated the importance of the IPA pathway in the IAA biosynthesis, sometimes almost 90% of the total IAA production (Theunis et al., 2004). IPA proceeds via indole-3-acetaldehyde (IAAld) to IAA through the indole-3-pyruvic acid decarboxylase (IpdC) and IAAld dehydrogenase activities, respectively (Spaepen et al., 2007). It has been proposed that the rate-limiting step of the IPA pathway is the conversion of IPA to IAAld, and some associated decarboxylases encoded by the *IpdC* gene have been characterized from several bacteria (Patten and Glick, 2002; Blaha et al., 2006; Baudoin et al., 2010). However, very recent studies focusing on the last step of the IPA pathway have evidenced the important role of aldehyde-dehydrogenase (ALDH) encoded by the bacterial gene *dhaS*, since almost 77% of the IAA production decreased when *dhaS* gene was repressed (Shao et al., 2015).

Although reports on bacterial synthesis of IAA are numerous, the regulation of IAA biosynthesis has been poorly documented. Recent research has elucidated a positive regulation of tryptophan in IAA synthesis as well as a positive-feedback-loop regulation by the end-product IAA of the IPA pathway (Spaepen and Vanderleyden, 2011). In this scenario, it would be feasible that exogenous tryptophan could enhance IAA biosynthesis by those vermicompost-borne bacteria able to produce IAA.

Natural sources of tryptophan include feedstuffs providing the adequate dietary supply of amino acids to meet animals' requirements, and sunflower-oil cake is one of the major protein sources for the livestock industry. It is the by-product in extracting of oil from sunflower seeds and, in terms of production, the 4th most important oil meal in the world (Oil World, 2011). Sunflower cake has a protein content of about 27%, with tryptophan comprising about 1.4% (Ramachandran et al., 2007).

The aim of this study was to elucidate whether or not an exogenous source of tryptophan was able to stimulate the bacterial biosynthesis of the auxin IAA during the vermicomposting of an organic agro-waste. For this, sunflower-oil cake or synthetic tryptophan were introduced into a biodegradation system of vermicomposting olive wastes. A novel degenerated set of primers targeting bacterial ALDH-encoding genes were performed from several ALDH proteins of plant-beneficial bacteria. A method involving ultrasound-assisted extraction of IAA and subsequent analysis by liquid chromatography–tandem mass spectrometry was also optimized in order to improve recovery. The effects of tryptophan on the vermicomposting process and influence of the resulting vermicompost on the bacterial microbiome of the pepper (*Capsicum annuum* L.) rhizosphere were evaluated by assessing the number and the expression of ribosomal and ALDH-encoding genes as well as the IAA content.

2. Materials and methods

2.1. Vermicomposting process

A 9-month-old vermicompost from a mixture of fresh olive waste and sheep manure (8:1 dry weight) was used as initial substrate (Vo) for vermicomposting. The main characteristics are described in Vivas et al. (2009). Sunflower oil cake (Sc) composition is reflected in Martin-Garcia et al. (2004) and tryptophan content of Sc (0.37%) was determined by alkali hydrolysis as described Ren et al. (2007).

Firstly, earthworm viability was tested in mixtures of Vo with increasing doses of Sc. After 5 days of exposure, toxicity was detected from a concentration of Sc of 10% (dry weight.). A 2% Sc dose (dry weight) was then selected to give the VSc treatment, that is, Vo mixed with 2% Sc. An amount of L-tryptophan (Sigma-Aldrich) equivalent to that supply by Sc in the VSc treatment was added to Vo to give the VT treatment. Vo alone was used as control (V). Each treatment was replicated three times.

A total of 200 gr (dry weight basis) of each substrate was placed in 1 L plastic containers made from 12-cm lengths of PVC pipe (12 cm internal diameter) with a fine nylon mesh held in place at the bottom. Ten grams, equivalent to 15 clitellated earthworms (*Eisenia foetida*), were added to each treatment. The moisture content of the substrates was maintained at 80–85% and the containers were kept in darkness at 25 °C throughout a 2-months vermicomposting period. Afterwards, earthworms were picked out by hand and substrates were air-dried outdoors. For the isolation of nucleic acids, subsamples of the initial and final substrates were immediately frozen in liquid nitrogen and then stored at −80 °C until molecular analyses were performed.

Electrical conductivity and pH was measured in 1:5 (dw/v) organic material/deionized water ratio (M.A.P.A., 1986).

2.2. Pot experiment

The experiment was performed in a controlled-environment greenhouse in 2 L plastic pots. Each pot contained 1000 g of undisturbed soil samples of a calcareous loam TypicXerorthent (Soil Survey Staff, 1999). The relevant characteristics of the soil are given in Moreno et al. (2011). As a means of reaching a soil organic C content of 35 g dry weight kg^{-1}, the soil was thoroughly mixed with the vermicompost at the corresponding rate. Unamended soil was used as a control (S). Pepper (*Capsicum annuum*, L., cv Melchor) seeds grown on vermiculite for 30 days, provided by Carmelo Ruiz (Department of Biochemistry and Molecular and Cellular Biology of Plants −ARNOBA Group- EEZ-CSIC), were transferred to the soil and grown in the greenhouse for 3 months. There were three replicates for each of the treatments assayed. Pots were arranged in randomized blocks and irrigated daily with distilled water to field capacity. Rhizosphere soil was collected in two steps. First, the root system was separated from the bulk soil by gently shaking, and then the soil remaining attached to the roots was separated from the roots by more vigorous shaking. Soil still adhering to the roots was removed using a sterile dissecting probe and collected as rhizosphere soil. Root-associated soil samples from each pot were placed in separate polyethylene plastic bags and immediately stored at −80 °C until molecular analyses was performed.

For vermicompost and soils, total organic carbon and total N content was determined by an elemental analyzer Leco TruSpec CN. Nitrogen was detected by the Dumas method: complete combustion of the sample at 950 °C with high-pressure oxygen, chemical reduction of N oxides to molecular N and analysis of this molecular N with a thermal conductivity detector (TCD). C was detected as carbon dioxide (from the sample combustion) by an infrared detector. The organic C was measured in an additional analysis, by combustion at 500 °C. The quantification was performed with certified standards from Leco, each of different concentration.

2.3. Nucleic acid isolation from soils and cDNA synthesis

For each sample of vermicompost or soil replicate, total DNA was separately extracted from four 0.25 g or 0.5 g subsamples, respectively, by the bead-beating method, following the manufacturer's instructions