Plant Growth Regul (2015) 75:483–492
DOI 10.1007/s10725-014-0011-0

ORIGINAL PAPER

Evidence of phytohormones and phenolic acids variability in garden-waste-derived vermicompost leachate, a well-known plant growth stimulant

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Received: 7 October 2014 / Accepted: 10 December 2014 / Published online: 18 December 2014

Abstract Cytokinins, auxins, abscisic acid, gibberellins (GAs) and brassinosteroids (BRs) as well as the phenolic acid content in three batches of vermicompost leachate (VCL) were quantified using ultra high performance liquid chromatography–tandem mass spectrometry. N^6-isopentenyladenine formed the major (60 %) proportion of the CK content while dihydrozeatin had the lowest (<0.02 %) concentration. Indole-3-acetic acid ranged from approximately 0.55–0.77 pmol/mL. A total of 18 GAs including bioactive forms and metabolic end products were observed in the VCL samples. Cathasterone had the highest (2,500–3,200 fg/mL) concentration while brassinolide was the lowest (1–5 fg/mL) abundant BRs found. Phenolic acids quantified were protocatechuic acid (3–3.6 μg/mL), *p*-hydroxybenzoic acid (2.5–2.8 μg/mL), *p*-coumaric acid (1–1.7 μg/mL) and ferulic acid (0–4 μg/mL). These results provide an indication of the rich diversity in natural PGRs and phytochemicals in VCL which may inevitably contribute to the numerous favorable physiological responses elicited by VCL application to plants.

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Keywords Abscisic acid · Biostimulant · Brassinosteroids · Cytokinins · Gibberellins · Phenolics

Abbreviations

ABA	Abscisic acid
BRs	Brassinosteroids
CKs	Cytokinins
*c*Z	*cis*-Zeatin
*c*Z9G	*cis*-Zeatin-9-glucoside
*c*Z*O*G	*cis*-Zeatin-*O*-glucoside
*c*ZR	*cis*-Zeatin riboside
*c*ZRMP	*cis*-Zeatin riboside-5′-monophosphate
*c*ZR*O*G	*cis*-Zeatin-*O*-glucoside riboside
DHZ	Dihydrozeatin
DHZ9G	Dihydrozeatin-9-glucoside
DHZ*O*G	Dihydrozeatin-*O*-glucoside
DHZR	Dihydrozeatin riboside
DHZRMP	Dihydrozeatin riboside-5′-monophosphate
DHZR*O*G	Dihydrozeatin-*O*-glucoside riboside
GAs	Gibberellins
IAA	Indole-3-acetic acid
IAC	Immunoaffinity chromatography
iP	N^6-Isopentenyladenine
iP9G	N^6-Isopentenyladenine-9-glucoside
iPR	N^6-Isopentenyladenosine
iPRMP	N^6-Isopentenyladenosine-5′-monophosphate
IPT	Isopentenyltransferase
MRM	Multiple reaction monitoring
PGR	Plant growth regulator
SPE	Solid-phase extraction
*t*Z	*trans*-Zeatin
*t*Z9G	*trans*-Zeatin-9-glucoside
*t*Z*O*G	*trans*-Zeatin-*O*-glucoside
*t*ZR	*trans*-Zeatin riboside
*t*ZRMP	*trans*-Zeatin riboside-5′-monophosphate

*t*ZR*O*G	*trans*-Zeatin-*O*-glucoside riboside
UHPLC	Ultra high performance liquid chromatography

Introduction

The increasing population has led to increased pressure on the food/agricultural sector. There is a need for efficient use of the available natural resources such as limited land and water for sustainable agricultural production (Tilman et al. 2002; Sharma et al. 2014). Intensive high-yield agriculture production and nutrient deficient soils depend on the application of inorganic chemical fertilizers to promote and sustain plant growth, development and yield. However, an increase in the use of inorganic fertilizers has been linked to severe environmental problems such as water and soil pollution. In addition, inorganic fertilizers are expensive and not easily accessible to many small-scale farmers in most developing countries (Sharma et al. 2014). Consequently, researchers are continuously exploring means of improving crop yield and quality without compromising environmental integrity or public health. Sustainable agricultural practices which entails the conservation of resources and the environment remain a viable and cheaper option to increase agricultural product output (Tilman et al. 2002). Such good agricultural practices determine the level of food production and, to a great extent, the state of the global environment. Application of organic biostimulants in low concentrations enhance plant growth and development and their use has increased globally (Theunissen et al. 2010; Sharma et al. 2014). These biostimulants often include diverse formulations of compounds and products such as microorganisms, plant growth regulators (PGRs), macroalgal extracts and vermicompost.

Different vermicompost formulations include their leachates, teas and other extracts. They are dark, odourless and nutrient-rich products obtained from a wide range of organic wastes using earthworms. Vermicompost are made by controlled aerobic and thermophilic biological transformation to attain the required pasteurization and a specified level of maturity (Campitelli and Ceppi 2008). The increasing acceptance of vermicompost products is due to numerous benefits including their usefulness in plant growth media and soil ameliorants (Theunissen et al. 2010; Aremu et al. 2012) as well as alleviation of nutrient deficiency (Arthur et al. 2012; Aremu et al. 2014) and other abiotic stress conditions (Chinsamy et al. 2013).

As is common with other biostimulants (Sharma et al. 2014), vermicompost leachate (VCL) are applied at low concentrations either as a soil drench or foliar spray to elicit various physiological responses. Thus, the numerous beneficial effects of VCL cannot be attributed to an increase in the supply of macro- and micro-nutrients, PGRs and other elicitor biomolecules are considered as the active ingredients. Increasing evidence supporting these assertions have been demonstrated (Tomati et al. 1988; Arthur et al. 2001; Suthar 2010; Singh et al. 2014). Recently, Zhang et al. (2014) provided the first mass spectrometric evidence of some isoprenoid cytokinins (CKs) in vermicompost tea. Despite using 12 representative standards from the different classes of PGRs, only CKs were quantified. Generally, extremely low concentrations and problems of interference with other plant phytochemicals have been major challenges for extraction, purification and the precise and accurate quantification of PGRs. The aim of the present study was to quantify the concentrations of PGRs such as CKs, auxins, abscisic acid (ABA), gibberellins (GAs) and brassinosteroids (BRs) that may be present in commercially produced garden-waste-derived VCL using ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis which is the most precise method currently available. In addition, the phenolic acid content in the VCL samples was evaluated. As a quality control measure, three batches of VCL were tested as disparity during production in parameters such as chemical, physical and biological composition amongst different vermicompost samples has been reported (Campitelli and Ceppi 2008; Pant et al. 2012).

Materials and methods

Source of vermicompost leachate

Three different manufactured batches of VCL were purchased from Wizzard Worms Commercial Company Ltd, Greytown, South Africa. According to the manufacturer, the VCL (pH 7.82) is derived from garden-waste (for example, vegetables) using the red earthworm *Eisenia fetida*. The macronutrients per dry matter include nitrogen (2.26 %), phosphorus (0.99 %), potassium (0.64 %), calcium (2.52 %) and sodium (631.03 mg/kg) as stated on the label. Further details on the preparation procedure of the VCL are available from the company (http://www.wizzardworms.co.za). It is marketed as a natural organic plant-promoting product.

Phytohormone quantification

Cytokinin quantification

The procedure for purification and quantification of CKs in the VCL samples were as described previously (Novák

et al. 2008) with slight modifications. Four replicates of 1 mL aliquots from each batch of VCL were extracted in 15 mL Bieleski buffer (60 % methanol, 25 % $CHCl_3$, 10 % HCOOH and 5 % H_2O). Deuterium-labeled CK internal standards (1 pmol) were added to samples to check recoveries during purification and to validate the results. The resultant supernatants were cleaned using an SCX cationic exchange column (Varian Inc., CA, USA). Thereafter, two purified CK factions (ribotides and other bases, ribosides and glycosides) were obtained using coupled Sephadex (Sigma–Aldrich, St. Louis, MA, USA) and reverse-phase (C18) columns (Waters, Milford, MA, USA). The obtained eluates were evaporated to dryness. For the qualitative and quantitative phytohormone analysis, the eluates from the two CK fractions were analyzed using UHPLC® equipped with a reverse-phase BEH C18 column (1.7 µm, 2.1 mm × 150 mm) (Milford, MA, USA), linked to a Xevo™ TQ MS triple quadrupole mass spectrometer with an electrospray interface (Waters MS Technologies, Manchester, UK). Further details on the conditions of the process was described by Novák et al. (2008).

Auxin quantification

Indole-3-acetic acid (IAA) content in the VCL samples was determined as described by Pěnčík et al. (2009). Four replicates (1 mL VCL) were taken from each batch for the analysis. An internal standard of 5 pmol $^{13}C_6$-IAA (Cambridge Isotopes Laboratories, Tewksbury, MA, USA) was added to each sample. After centrifugation at 20,000 rpm for 10 min, supernatant were diluted with 4 mL water, acidified with 1 M HCl to pH 2.7 and purified by SPE using C8 columns (Bond Elut, Varian). Samples were then evaporated to dryness, derivatized with diazomethane and processed by immunoaffinity purification based on anti-IAA polyclonal antibodies. Eluates were evaporated to dryness under the stream of liquid nitrogen and analyzed by UHPLC–MS/MS (Pěnčík et al. 2009).

Abscisic acid quantification

The ABA content in the VCL samples (1 mL) was purified using a modified method described by Yokoya et al. (2010). In order to check the recovery during purification and to validate the determination, 20 pmol of deuterium-labeled ABA ([(+)-3′,5′,5′,7′,7′,7′-$^2H_6$-ABA] synthesized as described by Prinsen et al. (1995)) was added to the samples. Thereafter, the VCL samples were directly purified by solid-phase extraction (SPE) on Oasis® HLB cartridges (60 mg, 3 mL, Waters, Milford, MA, USA). These were subsequently evaporated to dryness and derivatized by methylation with diazomethane. The samples were further subjected to ABA-specific immunoaffinity chromatography as described by Hradecká et al. (2007). The eluents were evaporated to dryness and quantified by UHPLC–MS/MS following the previously described procedures (Turečková et al. 2009).

Gibberellin quantification

The concentration of GAs in the VCL samples were quantified following the methods of Urbanová et al. (2013) with slight modifications. Four replicates of 1 mL aliquots from each batch of VCL were centrifuged for 10 min at 17,000 rpm and 4 °C. The pellet was further extracted overnight in 1 mL 80 % acetonitrile containing 5 % formic acid with the addition of 19 internal GA standards ([$^2H_2$]GA_1, [$^2H_2$]GA_3, [$^2H_2$]GA_4, [$^2H_2$]GA_5, [$^2H_2$]GA_7, [$^2H_2$]GA_8, [$^2H_2$]GA_9, [$^2H_2$]GA_{12}, [$^2H_2$]GA_{12}ald, [$^2H_2$]GA_{15}, [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{24}, [$^2H_2$]GA_{29}, [$^2H_2$]GA_{34}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_{51} and [$^2H_2$]GA_{53}; OlChemIm, Olomouc, Czech Republic). After centrifugation at 17,000 rpm and 4 °C for 10 min, the pellet was reextracted in 1 mL of extraction solvent for 1 h and centrifuged again. The supernatants derived from both extractions were combined and evaporated to dryness using vacuum concentrator (CentriVap® Acid-Resistant benchtop concentrator, LabconcoCorp., MO, USA) and the extract was further purified using Oasis® MAX cartridges (Waters, Milford, MA, USA). Dried eluates were reconstructed in the mobile phase and analyzed by UHPLC–MS/MS. Gibberellins were detected using multiple-reaction monitoring (MRM) mode of the transition of the precursor ion $[M–H]^-$ to the appropriate product ion.

Brassinosteroid quantification

The concentration of BRs in the VCL samples were quantified as described by Swaczynová et al. (2007) with slight modifications. Aliquots (1 mL) of the VCL samples were extracted overnight in ice-cold 80 % methanol with 30 pmol of [$^2H_3$]brassinolide, [$^2H_3$]castasterone, [$^2H_3$]24-*epi*brassinolide and [$^2H_3$]24-*epi*castasterone as internal standards (OlChemIm, Olomouc, Czech Republic). Thereafter, the samples were purified on polyamide SPE columns (Supelco, Bellefonte, PA, USA) and evaporated to dryness in vacuo. Dried eluates were reconstructed in the mobile phase and analyzed by an UHPLC–MS/MS system.

Phenolic acid quantification

The concentration of phenolic acids in the VCL samples were quantified as described by Gruz et al. (2008). Briefly, four replicates of 1 mL each were taken from three batches of the VCL. Deuterium-labeled internal standards of 4-hydroxybenzoic (2,3,5,6-$^2H_4$) and salicylic (3,4,5,6-$^2H_4$)

acids purchased from Cambridge Isotope Laboratories (Andover, MA, USA) were added at a final concentration of 10^{-5} mol/L to the extraction solvent prior to the homogenization. The supernatants were filtered by centrifugation at 3,500 rpm for 5 min through 0.45 μm nylon membrane filters (Micro-Spin™, All-tech, Deerfield, IL, USA) and analyzed using UHPLC–MS/MS system. Formic acid and methanol used for preparing mobile phases were purchased from MERCK (Darmstadt, Germany). Deionized water was prepared using a Simplicity 185 system (Millipore, Bedford, MA, USA).

Data analysis

The data for the CKs, ABA, GAs, BRs and phenolic acids were analyzed using Masslynx 4.1 software (Waters, Milford, MA, USA). Subsequently, the content of endogenous phytohormones and phenolic acids was determined by the standard isotope-dilution method in the VCL samples (Rittenberg and Foster 1940; Croker et al. 1994).

Results and discussion

While the various physiological responses mediated by VCL are extensively documented, there is limited data relating to the active compounds present in these products. The stimulatory effects have often been attributed to the presence of mineral elements, humic acid and PGR-like compounds in vermicompost (Tomati et al. 1988; Arthur et al. 2001; Campitelli and Ceppi 2008; Suthar 2010). Humic acid compounds influence the growth of plants by increasing anion and cation uptake, protein synthesis and the action of nitrate metabolism enzymes as well as enhancing macro- and micro-nutrient adsorption (Tomati et al. 1988). Despite the identification of humic acid compounds, increasing evidence strongly infers the presence of PGR being the main active compounds in VCL (Tomati et al. 1988; Arthur et al. 2001; Suthar 2010; Singh et al. 2014). Production of vermicompost involves the use of earthworms and evidence suggests that earthworms produce a considerable amount of PGR-like and other growth promoting substances in their body secretions. The vermiwash may contain CK, auxin, amino acid and vitamins as well as enzymes possibly derived from microbes associated with earthworms (Tomati et al. 1988). Positive identification of PGR in VCL is essential in order to justify and determine its feasibility in crop production (Suthar 2010). For the first time, the current study provides quantitative and qualitative evidence of the rich cocktail of PGR and phenolic acid present in VCL. Apart from ABA which was not detected in any of the VCL samples, different concentrations of CK, GAs and BRs were positively identified in the current study.

Cytokinin concentrations and role in vermicompost leachate

Only isoprenoid-type CKs were quantified in the three batches of VCL. In terms of the abundance of CK-types, iP > *c*Z > *t*Z > DHZ in all the three batches of VCL (Table 1). Among the four isoprenoid-type CK quantified, N^6-isopentenyladenine (iP) formed the major (60 %) proportion while dihydrozeatin (DHZ) had the lowest (<0.02 %) concentration (Table 1). The total CK content and individual CK-type was generally higher in batch 1 compared to the other two batches. Based on the function of the CKs, the nucleotide > ribosides > free bases > *O*-glucosides while *N*-glucosides were below detection limits in all three batches of VCL (Fig. 1). The critical role of CK in stimulating growth is well recognized. They are naturally occurring N^6-substituted adenine compounds that are essential in regulating several physiological and developmental processes such as shoot apical dominance, branching, chlorophyll production, senescence and root growth in plants (Sakakibara 2006). There is strong evidence that CKs are able to move among plants, other organisms and the different physical components of the biosphere and this may explain the frequent identification of CKs in many biostimulants used in agriculture (Sharma et al. 2014). There are several studies involving the application of VCL where CK-like activity has been demonstrated in plant species (Suthar 2010; Aremu et al. 2012; Arthur et al. 2012) and thus researchers have postulated that vermicompost possibly contains some levels of CKs (Tomati et al. 1988; Suthar 2010). Recent findings by Zhang et al. (2014) and Pant et al. (2012) identified 3 CKs in vermicompost tea. In contrast, 15 CK derivatives were identified in the three batches of VCL samples analyzed in the current study based on an UHPLC–MS/MS system (Table 1). These derivatives were all isoprenoid-type CKs. Isoprenoid CKs are more abundant than aromatic CKs in higher plants (Sakakibara 2006). Considering that the VCL samples were derived from green vegetable waste, the absence of aromatic CKs in the VCL samples is justified. This is in agreement with other researchers who also only reported isoprenoid CKs in vermicompost (Pant et al. 2012; Zhang et al. 2014).

Apart from the quantity of CKs in plant and biostimulants, their composition is also important in order to stimulate favorable biological activity. To ensure homeostasis, CKs are rapidly metabolized between the free bases and their corresponding nucleotides and nucleosides in a circular rather than unidirectional flow while conversion to *N*-glucosides is irreversible (Sakakibara 2006). In the

Table 1 Cytokinin content (fmol/mL) quantified in three batches of VCL

Cytokinins	Batch 1	Batch 2	Batch 3
*t*Z	4.33 ± 0.243	3.47 ± 0.406	3.55 ± 0.530
*t*ZR	0.98 ± 0.071	0.56 ± 0.017	0.57 ± 0.023
*t*ZRMP	3.87 ± 0.392	3.55 ± 0.487	3.42 ± 0.219
*t*ZOG	0.73 ± 0.101	0.54 ± 0.032	0.52 ± 0.032
*t*ZROG	0.03 ± 0.001	0.03 ± 0.001	0.03 ± 0.005
Total *t*Z-types	9.93 ± 0.590	8.15 ± 0.822	8.09 ± 0.578
*c*Z	11.89 ± 0.381	9.08 ± 0.660	8.47 ± 0.558
*c*ZR	5.60 ± 0.182	5.50 ± 0.388	5.43 ± 0.278
*c*ZRMP	107.96 ± 1.691	90.67 ± 10.776	85.10 ± 5.893
*c*ZOG	1.45 ± 0.143	1.12 ± 0.196	1.01 ± 0.117
*c*ZROG	1.68 ± 0.182	1.23 ± 0.197	1.38 ± 0.216
Total *c*Z-types	128.59 ± 1.500	107.60 ± 11.642	101.39 ± 6.301
DHZRMP	4.58 ± 0.199	4.93 ± 0.476	3.92 ± 0.304
DHZROG	0.10 ± 0.012	0.10 ± 0.009	0.07 ± 0.004
Total DHZ-types	4.68 ± 0.201	5.02 ± 0.482	3.99 ± 0.301
iP	20.35 ± 1.166	13.01 ± 0.453	15.90 ± 1.455
iPR	99.84 ± 7.595	57.45 ± 2.936	73.42 ± 7.130
iPRMP	94.90 ± 14.589	56.56 ± 9.381	64.93 ± 10.392
Total iP-types	215.08 ± 21.029	127.01 ± 9.908	154.25 ± 4.813

Results are presented as mean ± SEM (n = 4), <LOD = below limit of detection

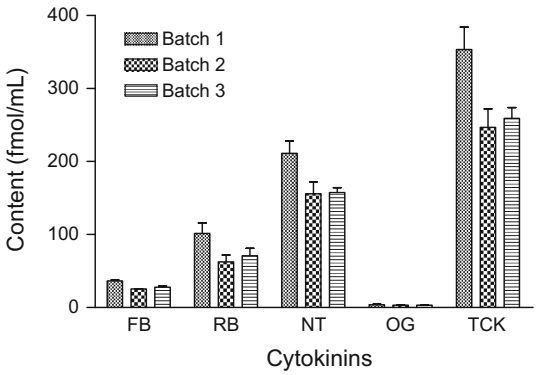

Fig. 1 Cytokinin content (fmol/mL) quantified in three batches of VCL. Results are presented as mean ± SEM (n = 4). *FB* free bases, *RB* ribosides, *NT* nucleotides, *OG* *O*-glucosides, *TCK* total cytokinins

current study, nucleotide, riboside, free bases and *O*-glucosides consisted approximately 61, 27, 11 and 1 %, respectively of the total CK pool (Fig. 1). Similar composition and abundance of the biological active CKs as well as lack of detection of the *N*-glucosides (deactivation product) was observed in macroalgae-derived Kelpak® (Stirk et al. 2004). Even though *N*-glucosides are often regarded as deactivation products, recent evidence has demonstrated certain degree of biological activity (Gajdošová 2011; Mik et al. 2011). Among the (well-recognized) biologically active free bases, iP > *c*Z > *t*Z > DHZ in the three batches of VCL with iP comprising approximately 50 %. Based on results from various bioassays, the free bases iP and *t*Z are considered as the most active forms (Sakakibara 2006) while *c*Z generally has little or no activity (Gajdošová 2011). However, both *t*Z and *c*Z exhibited similar biological activity in rice (Kudo et al. 2012). In the context of the current study, the bioactive CKs, especially iP and *t*Z may possibly explains the CK-like activity observed when different crops are treated with VCL.

Auxin concentrations and role in vermicompost leachate

Even though the applied protocol had the potential to quantify seven auxin conjugates, only IAA was detected in the three batches of VCL. Batch 2 (0.77 ± 0.041 pmol/mL) of the VCL had the highest IAA content while batch 1 and 3 had 0.63 ± 0.034 and 0.55 ± 0.058 pmol/mL, respectively. In addition to being the first phytohormones discovered, auxins are chemically diverse compounds possessing an aromatic system such as indole, phenyl or naphthalene ring with a side chain containing a carboxyl group attached. Generally, IAA is the most abundant naturally occurring auxin and is involved in several physiological processes in plants. As with other phytohormones, it is essential to use methodology that offers low detection limits and high selectivity for accurate quantification of auxins including IAA. The approach used in the current study afforded the quantification of IAA content in vermicompost for the first time. However, the quantification of

IAA amino acid conjugates was not achieved which may be attributed to their relatively lower content when compared to IAA.

Although IAA was the only auxin compound quantified in the current study, it is noteworthy that the concentration (0.55–0.77 pmol/mL) was relatively high when compared to individual CK bases such as *t*Z (3–4 fmol/mL) and iP (13–20 fmol/mL). As an active form of auxin, the quantity of IAA present in the tested VCL is an indication of the importance of IAA as a major active ingredient in VCL and explains the auxin-like activity often observed with VCL-treated plants (Aremu et al. 2012; Pant et al. 2012). For example, substances extracted from earthworm compost induced lateral root growth in maize plants by stimulating plasma membrane H+-ATPase activity, thus producing similar effects associated with the exogenous application of IAA (Façanha et al. 2002). Furthermore, IAA activity was detected in the humic acid structure (Canellas et al. 2011). In another study, the induction of lateral root initiation by vermicompost-derived humic substances in *Arabidopsis* was related to the activation of the transcription of some auxin responsive genes (Trevisan et al. 2010). Together with the aforementioned studies, our findings confirm the presence of IAA in vermicompost.

Gibberellin concentrations and role in vermicompost leachate

About 136 GAs (GA_1–GA_{136}) have been identified in plants, fungi and bacteria (Piotrowska and Bajguz 2011). As previously hypothesized by several authors (Tomati et al. 1988; Singh et al. 2014), the present of pool of GAs in VCL was positively established in the current study. Previously, Pant et al. (2012) reported varying concentrations of three GAs (GA_4, GA_{24} and GA_{34}) in vermicompost derived from different sources. In the current study, a total of 18 GAs consisting of biologically active forms, biosynthetic precursors or deactivation metabolites were identified in the VCL samples evaluated (Table 2). The total GA content ranged from 552 (batch 1) to 656 pg/mL (batch 3) in the VCL samples (Table 2). As depicted in Fig. 2, the final metabolic products of GAs were generally higher than the bioactive forms. In all three batches of VCL, GA_{51} constituted approximately 40 % of the total GA content while GA_6 (the most abundant active GA form detected) constituted approximately 2 % of the total GA pool.

Recently, Stirk et al. (2014) reported an equal number of GAs in the macroalgae-derived Kelpak®. However, different types of GAs constituted the most abundant biologically active GA forms in Kelpak® (GA_4) and VCL (GA_6).

Table 2 Gibberellins content (pg/mL) quantified in three batches of VCL

Gibberellin	Batch 1	Batch 2	Batch 3
GA_1	**8.41 ± 0.274**	**10.30 ± 0.187**	**12.14 ± 0.615**
GA_3	**8.78 ± 0.124**	**8.62 ± 0.111**	**8.31 ± 0.102**
GA_4	**0.10 ± 0.003**	**0.11 ± 0.006**	**0.09 ± 0.004**
GA_5	**8.17 ± 0.220**	**8.93 ± 0.293**	**10.37 ± 0.443**
GA_6	**15.39 ± 1.049**	**14.05 ± 0.788**	**13.18 ± 1.098**
GA_7	**2.37 ± 0.091**	**1.63 ± 0.125**	**1.83 ± 0.189**
GA_8	*39.79 ± 3.445*	*37.42 ± 2.390*	*43.39 ± 1.006*
GA_9	22.64 ± 0.666	35.54 ± 1.800	26.38 ± 3.156
GA_{13}	*1.34 ± 0.150*	*1.43 ± 0.132*	*0.85 ± 0.041*
GA_{15}	61.12 ± 3.582	52.89 ± 3.353	25.59 ± 1.963
GA_{19}	7.67 ± 0.157	11.26 ± 0.500	8.98 ± 0.366
GA_{20}	23.24 ± 1.971	8.42 ± 0.431	4.65 ± 0.032
GA_{24}	38.99 ± 1.549	35.19 ± 2.898	38.03 ± 3.147
GA_{29}	*37.46 ± 1.708*	*24.72 ± 1.552*	*31.18 ± 1.497*
GA_{34}	*8.74 ± 0.481*	*8.53 ± 0.181*	*7.28 ± 0.305*
GA_{44}	47.16 ± 2.118	73.50 ± 4.880	132.15 ± 3.908
GA_{51}	*214.85 ± 5.875*	*298.44 ± 16.018*	*276.02 ± 5.443*
GA_{53}	6.18 ± 0.498	21.96 ± 1.815	15.88 ± 0.504

Results are presented as mean ± SEM (n = 4), <LOD = below limit of detection. Bioactive are in bold font; final metabolic products (catabolites) are in italics and other GAs are precursor forms

$GA_{12\text{-aldehyde}}$ (GA_{12ald}) and GA_{12} are the first GAs in the biosynthetic pathway and were below the detection limit

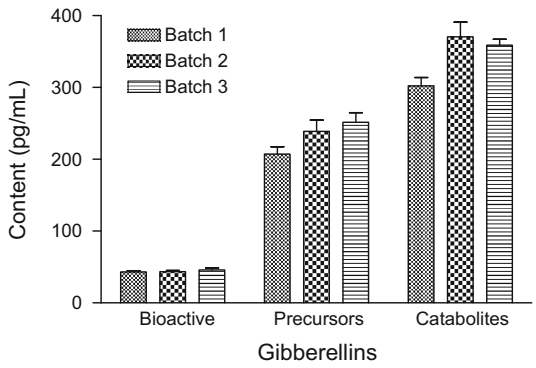

Fig. 2 Gibberellins content (pg/mL) quantified in three batches of VCL. Results are presented as mean ± SEM (n = 4). Bioactive = GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7; precursor forms = GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{44} and GA_{53} while the final metabolic products (catabolites) = GA_8, GA_{13}, GA_{29} GA_{34} and GA_{51}. GA_{12}-aldehyde (GA_{12}ald) and GA_{12} are the first GAs in the biosynthetic pathway and were found below the detection limit

In the current study, the majority (approximately 40 %) of the total GA content was GA_{51} (catabolic/deactivation product), while biologically active forms (GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7) were <10 % of the total GA pool in the VCL samples. Similar low concentration of biologically

active GAs (<3 %) was observed in Kelpak® (Stirk et al. 2014). Nevertheless, the possibility of the conversion of GA precursors (for e.g. GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{44} and GA_{53}) into active GAs once taken up by the plant has been suggested (Piotrowska and Bajguz 2011).

GAs are tetracyclic diterpenoids that act at all stages in the plant life-cycle including promoting germination, hypocotyl elongation, organ growth, greening of leaves, flowering and flower and seed development (Piotrowska and Bajguz 2011). The vital role of GAs on alleviating biotic (Iqbal et al. 2011) and abiotic stress such as salinity has also been highlighted (Iqbal et al. 2014). Pre-treatment of mung bean with GA_3 (0.1–10 μM) effectively alleviated the metabolic and biochemical alterations imposed by NaCl salinity (Chakrabarti and Mukherji 2003). As a demonstration of GA-like activity of VCL, bean seedlings treated with either VCL (1:10 v/v dilution) or GA_3 (10^{-5} M) had significantly longer seedling axes than the control (Singh et al. 2014). Both VCL and GA_3 (10^{-5} M) also increased seedling fresh weight when compared to the control. Proline accumulation which is a metabolic response to abiotic stress has been shown to be influenced by the presence of VCL in different crops (Chinsamy et al. 2013; Singh et al. 2014). Recently, the beneficial role of VCL under limited-nutrient conditions in greenhouse grown tomato (Arthur et al. 2012) and three medicinal plant species (Aremu et al. 2014) was demonstrated. These responses of VCL clearly agree with the ability of GAs to increase the photosynthetic potential of plants under stress resulting in more photosynthate production (source). This in turn enhances the sink strength (Iqbal et al. 2011).

Brassinosteroid concentrations and role in vermicompost leachate

Even though BRs (as with other PGRs) are present in low concentrations, our present methodology operating with relatively low detection limits successfully determined six BR types in the VCL samples (Table 3). BRs occurred in lower concentrations (fg/mL) compared to CKs and GAs (pg/mL). Among the six types of BRs detected, cathasterone (CT) had the highest (2,500–3,200 fg/mL) concentration while brassinolide (BL) was generally present in the lowest (1–5 fg/mL) quantity (Table 3). Batch 1 of the VCL sample had the highest concentration of BL, teasterone (TE) and 28-homocastasterone (homoCS) while typhasterol (TY) was most abundant in batch 3. No significant difference in the level of CT and castasterone (CS) was found in both batch 1 and 3 samples (about 3 pg/mL for CT and cca 40 pg/mL of CS). CS and BL are the most important naturally occurring BRs due to their wide distribution and potent biological activity (Bajguz and Tretyn 2003). While the VCL had ≤1 % BL, CS accounted for about 80 % of the total BRs quantified. Generally, the concentration of BRs quantified in these VCL samples were of significant lower magnitude when compared to values reported for a number of plant species (Bajguz and Tretyn 2003) including microalgae. The level of BRs in Kelpak® (biostimulant made from seaweeds) was also considerably higher than in VCL samples (Stirk et al. 2014). A possibly explanation for the low BR content analyzed in the present study is that VCL is a processed by-product of vegetative organs of green vegetable waste. Beside the loss which may have occurred during the processing, vegetative tissues are known to contain lower levels of BRs than reproductive tissues such as pollen and immature seeds (Bajguz and Tretyn 2003). However, BRs are effective at very low concentrations (Piotrowska and Bajguz 2011) and thus, VCL elicit BR-like physiological effects despite the low concentrations observed in the current study.

Among the known physiological responses of BRs are the promotion of cell division, elongation and influence on stem and root growth as well as floral initiation including flower and fruit development (Piotrowska and Bajguz 2011). In addition, BRs protect plants from abiotic and biotic stresses (Divi and Krishna 2009). Evidence of similar physiological effects have been observed in plants treated with VCL. For example, application of VCL improved root, shoot, stem and leaf growth, the development of tomatoes and the accumulation of proline when grown under elevated saline conditions (Chinsamy et al. 2013). BRs are also known to induce salinity tolerance via regulation of proline metabolism in plants (Iqbal et al. 2014). The reduction in leaf length caused by phosphorus and potassium deficiency in greenhouse grown *Eucomis autumnalis* was successfully reversed by addition of VCL as well as increasing the number of leaves in phosphorus-deficient *Tulbaghia ludwigiana* (Aremu et al. 2014). From these aforementioned examples, there are similarities in growth and physiological effects obtained with exogenous BR application and VCL. Thus the presence of BRs in VCL may partly account for some of the numerous beneficial responses elicited with VCL application especially considering the BRs remain effective at low concentrations. While it is clear that BRs have the ability to enhance yield and stress tolerance in plants, the high cost of synthetic BRs together with the variability of results has discouraged their use in agriculture (Divi and Krishna 2009). Thus, the use of VCL may possibly provide an alternative and cheaper source of BRs easily accessible to both commercial and small-scale farmers.

Table 3 Brassinosteroid content (fg/mL) quantified in three batches of VCL

Brassinosteroid	Batch 1	Batch 2	Batch 3
Brassinolide	5.29 ± 0.347	2.74 ± 0.121	1.40 ± 0.112
Castasterone	46.37 ± 3.338	35.24 ± 2.183	46.96 ± 3.570
Teasterone	162.14 ± 10.168	140.66 ± 12.445	114.91 ± 4.648
Typhasterol	186.27 ± 5.544	221.15 ± 15.904	341.22 ± 22.942
28-Homocastasterone	162.72 ± 8.951	87.87 ± 2.948	89.73 ± 1.768
Cathasterone	3,246.97 ± 102.099	3,248.22 ± 37.820	2,537 ± 134.553

Results are presented as mean ± SEM (n = 4)

12 BRs were below limit of detection in the three batches of VCL

Phenolic acid concentration in vermicompost leachate

In the three batches of VCL, four types of phenolic acids including protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and ferulic acid were quantified (Fig. 3). While the concentrations of protocatechuic acid and *p*-hydroxybenzoic acid were relatively similar among the three batches of VCL, the amount of *p*-coumaric acid and ferulic acid fluctuated considerably with ferulic acid not detected in batch 2 of the VCL (Fig. 3). Phenolic compounds have various roles in plants including influencing the formation of organs such as roots and shoots and protection from oxidative stress (Balasundram et al. 2006). While the effect of vermicompost on the composition of phenolic compounds in plants has been highlighted (Theunissen et al. 2010), the current findings are the first report that VCL actually contain four phenolic acids. This is in agreement with the perception by Balasundram et al. (2006) regarding the occurrence and potential uses of phenolic compounds as agro-industrial by-products. These phytochemicals often form part of the natural plant defence system against infection and microbial invasions. Thus the presence of such phenolic acids in VCL may possibly contribute to their effectiveness against both biotic and abiotic stresses. Even though phenolic compounds such as caffeic acid, ferulic acid, protocatechuic acid, *p*-hydrobenzoic acid and vanillic acid are identified as potential allelopathic agents that inhibit growth, such chemicals at certain lower concentrations may also elicit stimulatory effects on different plant organs (Wu et al. 2007; Hussain et al. 2014). Perhaps, this explains the generally low (≤4 µg/mL) concentrations of the four phenolic acids quantified in these VCL samples. In addition, VCL increased the phenolic content in different plants (Aremu et al. 2014; Singh et al. 2014). The higher levels of total phenolics observed with vermicompost application would probably explain why plants grown under vermicompost had fewer attacks by arthropod pests, and better resistance to disease compared with plants that receive inorganic fertilizers (Theunissen et al. 2010). From the human health perspective, the ability of VCL to stimulate accumulation of phenolic compounds (antioxidants) is commendable (Balasundram et al. 2006; Theunissen et al. 2010; Aremu et al. 2014).

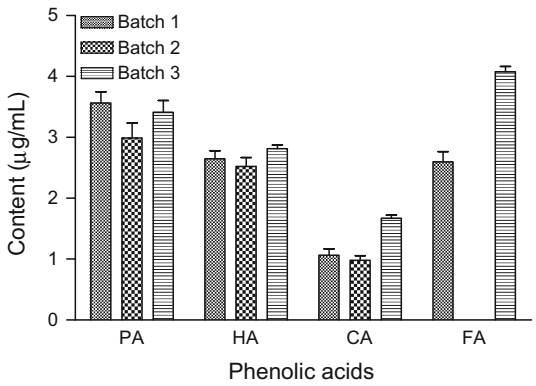

Fig. 3 Phenolic acid concentrations in three batches of VCL. Results are presented as mean ± SEM (n = 4). *PA* protocatechuic acid, *HA* *p*-hydroxybenzoic acid, *CA* *p*-coumaric acid and *FA* ferulic acid

Variations in phytohormones and phytochemicals in the three batches of vermicompost leachate

There is disparity in parameters such as chemical, physical and biological composition amongst different vermicompost samples and there is a need to devise tools for appropriate quality characterization of vermicompost (Campitelli and Ceppi 2008). Thus, the possible batch to batch variations in the tested VCL samples were evaluated as a quality control measure in the present study. Batch 1 sample had the highest level of CK and BR derivatives among the three batches (Table 1 and 3). In terms of the GA abundance, 8 GAs were highest in batch 1, 6 GAs in batch 2 and 4 GAs batch 3 (Table 2). Batch 2 also had the highest level of IAA. On the contrary, majority of the phenolic acids were highest in batch 3, with the exception of protocatechuic acid (batch 1). Similarly, vermicompost extracts from five different commercially produced

formulations had varying nutrient extraction efficiencies, microbial activity, phytohormones and total nutrient content (Pant et al. 2012). Inevitably, these differences significantly influenced the growth and mineral nutrient status of *Brassica rapa*. Despite the similar origin for all the batches of VCL used in the present study, variations in phytohormones and phenolic acids were prominent. The observed variations may be attributed to composition of the green vegetable waste used for the production of the VCL. In as much as possible, it will be necessary to use similar plant material for production of the VCL in order to minimize variability and guarantee the quality of the product.

Conclusions

The current study provide a quantitative evident of CKs, IAA, 18 GAs and 6 BRs in VCL. The cocktail of natural PGRs and phenolic acids present in VCL act in diverse manners and contribute to the numerous physiological responses such as enhanced growth and yield as well as improved stress responses to various biotic and abiotic stresses observed in VCL-treated plants. However, the observed variability in concentrations of PGRs and phenolic acids among the different batches of VCL is an indication more intense quality control measures during production of these products before it can be accepted as an affordable biostimulant.

Acknowledgments The Claude Leon Foundation, University of KwaZulu-Natal, and National Research Foundation, South Africa are thanked for financial support. This work was also financed by the Ministry of Education, Youth and Sport of the Czech Republic (the Program "Návrat" for Research, Development, and Innovations, no. LK21306), the Czech Science Foundation (Grant 14-34792S) and by the National Program for Sustainability (Grant. LO1204). We thank to Hana Martínková, Jarmila Greplová, Marie Vitásková and Andrea Novotná for excellent technical assistance.

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