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Article

Potato yield and metabolic changes by use of biofertilizer containing L-glutamic acid

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Abstract

The identification of natural bioactive sources capable of enhancing yield gains on agriculture is a tool to promote sustainability. In this study, the effect of foliar applications of sugarcane molasses fermented by the bacteria *Corynebacterium glutamicum*, containing 30% (w/v) of the amino acid L-glutamic acid was tested on organically grown potato for two seasons. Four foliar applications at intervals of ten days were made using solutions with different concentrations of the fermented broth (0.2, 0.4, 0.6, 0.8 mL L^{-1}). Total and commercial potato yield and biochemical alterations on leaves at 50 and 60 days after planting were determined. The applications of the fermented broth promoted yield gains and changes in chlorophylls, carotenoids, nitrate reductase enzyme (EC 1.6.6.1) activity, delaying the senescence and improving the free amino acid and soluble protein content of leaves. It was concluded that the fermented broth was able of improving potato yield by stimulating the nitrate reductase activity.

Keywords: *Solanum tuberosum*; *Corynebacterium glutamicum*; organic production; biofertilizer; amino acid

Introduction

One of the biggest challenges for the agriculture, which faces the growing worldwide food demand, is the establishment of sustainable production systems, the context in which natural products with a potential bioactive effect, such as amino acids, could contribute (Posmyk & Szafranska, 2016).

The amino acids applied to the plants could be considered as a plant biostimulant, regarding crop quality traits and yield (du Jardin et al., 2015). However, the mode of action of biostimulants as a class of products, for instance, the amino acids, is often unclear and difficult to be identified (Nardi et al., 2016). Regarding the Brazilian regulation, this class of products is defined as biofertilizers, natural sources that can act directly or indirectly on all or parts of the cultivated plants, enhancing their yield (Brasil, 2014).

Among biotechnological techniques, the fermentation of sugarcane molasses by the bacteria *Corynebacterium glutamicum* is known as an efficient process for obtaining the amino acid L-glutamic acid (Shyamkumar et al., 2014). The absorption of L-glutamic acid by foliar applications was reported by Beale et al. (1975) using C^{14} isotope. The authors proved the uptake and role of that amino acid on chlorophyll biosynthesis pathway by being incorporated into

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the aminolevulinic acid (ALA) on leaves of barley.

The benefits of using L-glutamic acid were reported by Sun & Hong (2010) on in vitro organogenesis of *Leymus chicness*, and by Lei et al., (2015) who stated that the Poli (y-glutamic acid) obtained by fermentation, promoted tolerance to abiotic stresses. Also, Calvo et al. (2014) reported that the exogenous amino acid application might promote nitrogen assimilation in plants by a coordinated regulation of C and N metabolism.

However, results from the use of fermented broth containing L-glutamic acid on the agriculture yield, and their correlation to the metabolic changes on field grown plants, remain scarce.

Changes in chlorophylls, carotenoids, free amino acids, soluble proteins and in the activity of enzymes in plant tissues could be indicators of the bioactivity of the fermented broth. Thus, the objective of this study was to evaluate the yield and the evaluation of the physiological effect of different concentrations of sugar cane broth containing 30% (w/v) of the L-glutamic acid released by bacteria in foliar applications on potato (*Solanum tuberosum* L.) plants grown in an organic production system for two seasons. By considering that potato is the fourth among the most consumed foods in the world (FAO, 2015), the development of efficient natural tools for organic potato management is strategic to a sustainable approach to food production.

Material and Methods

The potato cultivation was conducted for two seasons (2012 and 2013) at the research area of organic horticulture, where an organic production system was implemented 10 years ago, at the Federal University of Paraná, Paraná State, south of Brazil, between the geographic coordinates of 25° South latitude and 49°06' W longitude at 920 meters above sea level. The climate in the area is temperate type Cfb according to Köppen. The soil was classified as Oxisol.

The soil chemical analysis at the layer of 0-20 cm indicates the following average values: pH ($CaCl_2$) = 5.55; pH SMP= 6.0; Al^{+3}= 0; $H^{+}+Al^{+3}$= 5.50 cmolc dm^{-3}; Ca^{2+}= 9.85 cmolc dm^{-3}; Mg^{2+}= 9.8 cmolc dm^{-3}; K^{+}= 0.54 cmolc dm^{-3}; P= 42.6 mg dm^{-3}; C= 32.5 g dm^{-3}; percent nutrient saturation =72% and CEC= 15.54 cmolc dm^{-3}. The soil preparation in both seasons was done 20 days before tubers planting, with fertilization consisting of 200 kg ha-1 of magnesium thermophosphate (Yoorin®, with 17% P_2O_5) and 4 t ha^{-1} of organic compost, which the following average values: C = 30.3 g kg^{-1}; N= 30.3 g Kg^{-1}; P = 8.5 g kg^{-1}; K = 6.6 g kg^{-1}; Ca = 8.1 g kg^{-1}; Mg = 4.1 g kg^{-1}. Soil fertilization was done according Brazilian regulation for organic agriculture.

The first planting was carried out on October 8, 2011, and the second on September 2, 2012, in the adjacent areas. The plots had 3.2 m^2 with ten tubers linearly arranged with a spacing of 0.40 m between them, and 0.80 m between rows.

In both plantings, tubers of the cultivar 'Crystal' (Embrapa – Brazilian Agricultural Research Incorporation) with satisfactory budding were used. This cultivar is recommended for organic production due to their tolerance to late blight disease (*Phytophthora infestans*).

The experiments were set in a completely randomized design, with four replications. The treatments consisted of the foliar application of a solution with four concentrations (0.2, 0.4, 0.6, 0.8 mL L^{-1}) of sugarcane molasses fermented by the bacterium *Corynebacterium glutamicum*, containing 30% (w/v) of the amino acid L-glutamic acid (Microquimica Indústrias Químicas LTDA). The nutrient concentration on fermented broth was of 50 g L^{-1} (4% w/v) of amide nitrogen from amino acid released by bacteria, and 225 g L^{-1} of organic carbon (18% w/v) from sugarcane molasses, which was used according to the Brazilian organic regulations that prohibit synthetic nitrogen or amino acid sources.

The applications of fermented broth and the control sprayed with distilled water were performed using a pressurized CO_2 sprayer with constant pressure (125 psi) at the volume equivalent to 280 L ha^{-1}.

Four foliar applications were done with ten-day interval, starting 27 days after planting in both seasons.

Harvest in both seasons was done 97

days after planting when the aerial part of plants was completely senescent. All plants of each plot were collected for number of tubers, total yield, and commercial standard yield quantification. Yield was calculated by extrapolating the averages of plots to a population of 30.000 plants ha^{-1}, and the tubers with transverse diameter higher than 40mm were considered as a commercial standard.

In the second season, leaf samples were collected for biochemical determinations 50 days after planting, corresponding to the third day after the third foliar application, and again, 60 days after planting, corresponding to the third day after the fourth application.

In each sampling, which occurred from 9 to 10 a.m., two plants were used for each repetition, removing two full leaves of the middle third of each plant, which were immediately frozen. The following biochemical variables were determined: chlorophylls and carotenoids, the activity of nitrate reductase (EC 1.6.6.1), free amino acids and total soluble proteins in leaves.

Chlorophylls and carotenoids determinations were performed according to Pompelli et al. (2013).

The activity of the enzyme nitrate reductase (EC 1.6.6.1) was determined according to Jaworski (1971), with adaptations, as it follows: 1.0 g of fresh leaf sample was macerated in 8.0 ml of K phosphate buffer, pH 7.5. After that, the sample was incubated in water bath for 1 hour at 37°C with stirring in the dark. After incubation, 1 mL of sulfanilamide (Sigma-Aldrich®) was added, and 1 ml of a-naphthylamide (Sigma-Aldrich®), leaving at rest for 5 minutes. Then, it was filtered, and spectrophotometric reading was performed at 540 nm, with the amount of the formed nitrite compared with a standard curve of sodium nitrite ($NaNO_2$).

The free amino acids were determined by spectrophotometer readings at 570 nm according to Winters et al. (2002), comparing the readings with a standard curve of glutamine and asparagine.

Soluble proteins were extracted according to Du et al. (2010) with modification: phosphate buffer pH 7.5 and 100 mM, with the addition of 1 mM EDTA, 3 mM 1,4-dithiothreitol (DTT), 4% polyvinylpyrrolidone (PVP) (w/v) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The solution was homogenized by vortexing for 10 seconds at low speed, and after, centrifuged at 9.000 x g for 15 min. The supernatant was collected for measuring at 595 nm. The standard curve was built with bovine serum albumin (BSA) at 0.2% (w/v).

Data were submitted to analysis of variance. If they were significant, they were submitted to regression analysis. Also, the exact fermented broth concentration related to the measured variables was obtained from the first derivative of the regression equation, equating it to zero. Software ASSISTAT version 7.7 Beta was used in the study.

Results and Discussion

Foliar sprays of the sugarcane fermented broth containing 30% (w/v) of L-glutamic acid, has improved potato total yield in both seasons. The improvement in the total yield has resulted in commercial standard yield gains, represented by tubers with transverse diameter greater than 40mm (Figure 1). In the first season, the fermented broth concentrations of 0.338 and 0.364 mL L^{-1} determined highest commercial and total yield gains, respectively. In the second season, the concentrations of 0.320 and 0.309 mL L^{-1} showed highest commercial and total yield gains, respectively.

The differences in the number of the tubers of the plants showed low coefficient of determination values in both seasons, varying from 16 to 19 tubers per plant in the first season, and from 13 to 16 in the second, indicating that the yield gains occurred due to the better growth of tubers, not due to the improvement in the number of tubers.

The tuber initiation is driven by environmental factors such as photoperiod and temperature (Aksenova et al., 2012), in which interaction with the plants depends on the planting period and climate (Augustin et al., 2012).

The potato tuber bulking depends on a hormone controlling cell division and expansion (Aksenova et al., 2012), and sucrose and amino acids accumulation by source – sink regulation.

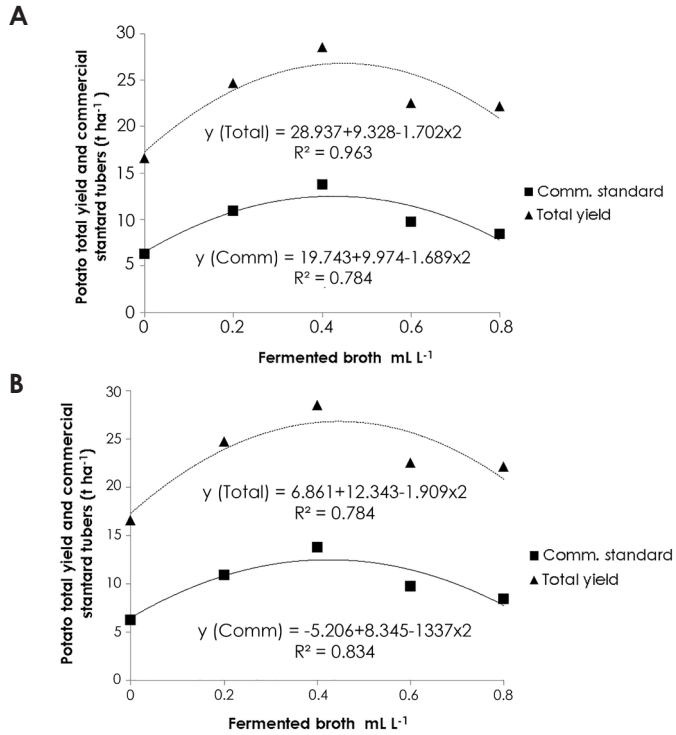

Figure 1. Total yield, yield of commercial standard (tubers with transverse diameter greater than 40mm) of organic potato 'Cristal', related to foliar applications of the sugarcane fermented broth containing 30% (w/v) of L-glutamic acid in two seasons (A-2012; B-2013).

Furthermore, it also depends on the amino acid synthesis in leaves and its uploads to phloem (Katouh et al., 2015).

The endogenous L-glutamic acid is a precursor of chlorophyll synthesis in developing leaves. The changes in the content of chlorophylls in potato leaves by fermented broth foliar applications (Figure 2) indicate the absorption and metabolization of L-glutamic acid, corroborating to the results of Beale et al. (1975), who proved the foliar absorption of L-glutamic acid and its role in chlorophyll biosynthesis.

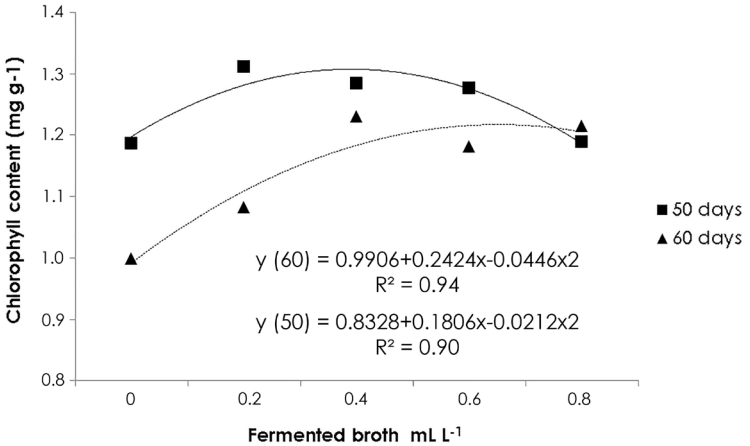

Figure 2. Chlorophyll content in potato leaves at 50 and 60 days after planting, related to foliar applications of the sugarcane fermented broth containing 30%(w/v) of L-glutamic acid.

The chlorophylls content at 50 days after planting (DAP), around the maximum leaf expansion stage of the potato plants (Zanon et al., 2013), was maximized by the fermented broth concentration of 0.234 mL L^{-1}, and on 60 DAP by 0.367 mL L^{-1}, suggesting that the phenological stage may interfere on the effect of fermented broth related to the chlorophylls synthesis (Figure 2).

At 60 DAP, the chlorophyll content of the

untreated plants was of 0.998 mg g^{-1}, lower than that found at 50 DAP (1.187 mg g^{-1}), indicating the onset of leaf senescence, characterized by biochemical changes involving the degradation of chlorophylls (Woo et al., 2013). At 60 DAP, after four applications, the effect of the fermented broth on the delay of chlorophyll degradation was well characterized by the increasing in their content related to the applied concentration.

This senescence delay effect is corroborated by the alterations on carotenoid content of potato leaves (Figure 3), which showed an understated alteration at 50 days after planting, when the plants were at the maximum vegetative development. However, in the control treatment, the carotenoids were increased at 60 days after planting by the senescence, and significantly decreased by fermented broth applications, highlighting 0.278 mL L^{-1} concentration, which determined the lowest content of carotenoids in potato leaves.

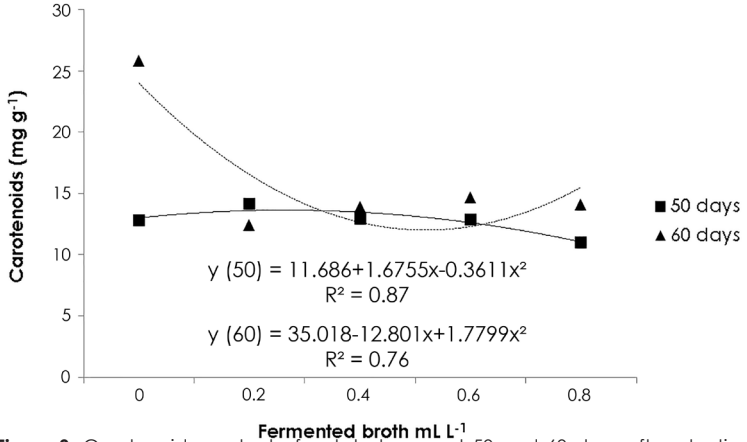

Figure 3. Carotenoids content of potato leaves at 50 and 60 days after planting related to foliar applications of the sugarcane fermented broth containing 30%(w/v) of L-glutamic acid.

The changes in the chlorophylls and carotenoids in tissues of potato leaves at early senescence could be related to the N metabolism, since the senescence could be regulated by C/N balance where the free amino acid concentrations were high in young leaves and progressively decreased in the senescent leaves (Agüera et al., 2010). Therefore, the changes in the content of free amino acid in potato leaves by the fermented broth applications (figure 4) somehow explain the effect on the senescence delay, mostly at concentration of 0.486 mL L^{-1}, which better improved the free amino acid content of leaves at 60 DAP.

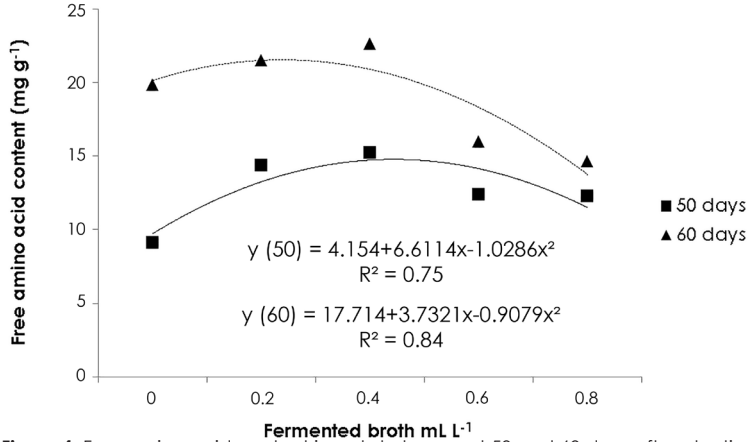

Figure 4. Free amino acid content in potato leaves at 50 and 60 days after planting related to foliar applications of the sugarcane fermented broth containing 30%(w/v) of L-glutamic acid.

The activity of nitrate reductase (Figure 5), the enzyme that acts on the pathway of amino acid synthesis showed a quadratic distribution and the free amino acid content, as well. In addition, the total soluble proteins of the leaves (Figure 6), also showed a quadratic response by the fermented broth concentrations increment, indicating the interaction among the enzyme activity and their consequent products: amino acids and proteins.

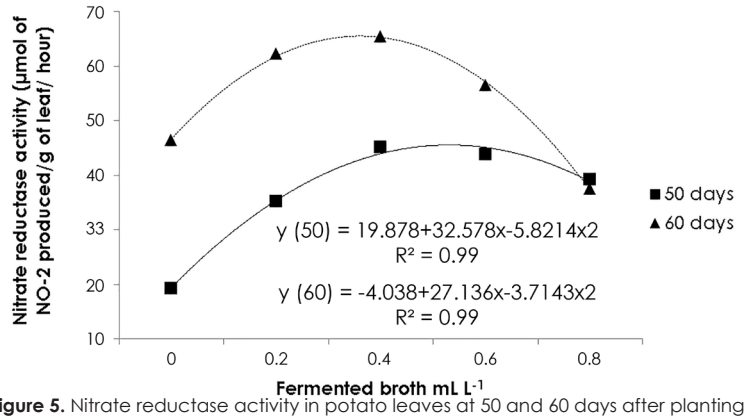

Figure 5. Nitrate reductase activity in potato leaves at 50 and 60 days after planting related to foliar applications of the sugarcane fermented broth containing 30%(w/v) of L-glutamic acid.

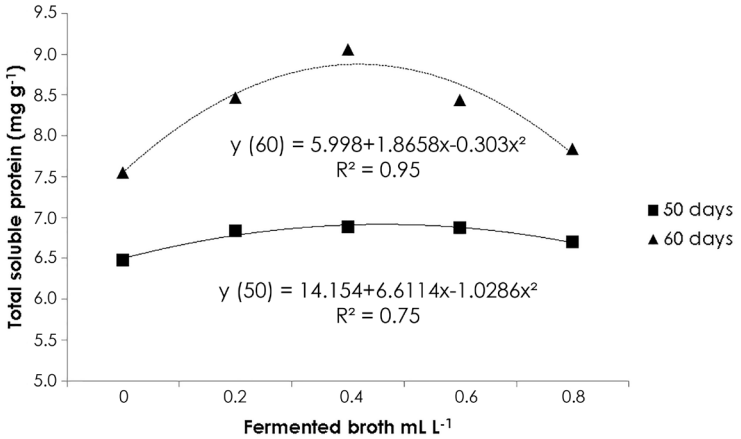

Figure 6. Total soluble protein content of potato leaves at 50 and 60 days after planting related to foliar applications of the sugarcane fermented broth containing 30%(w/v) of L-glutamic acid.

The changes in the contents of the chlorophylls (Figure 2) and carotenoids in the leaves (Figure 3), the changes in the nitrate reductase activity (Figure 5) and the alterations in free amino acids (Figure 4) and soluble proteins in leaves (Figure 6), indicate the foliar uptake and the role of sugarcane fermented broth containing 30% (w/v) of the amino acid L-glutamic acid, with consequent effect on the total and commercial potato yield at two consecutive seasons (Figure 1).

It should be mentioned that the concentrations of 0.338 and 0.32 mL L^{-1} showed the highest commercial yields gains in the first and second seasons, respectively. On the other hand, the increase in concentrations above those, gradually reduced the yield, indicating a possible negative feedback for chlorophylls and free amino acid synthesis.

The development and functioning of the photosynthetic system of plants is dependent upon the nitrogen (N) assimilation. In the nitrate (NO_3^-) assimilation, N is converted to a more energetic form, nitrite (NO_2^-), catalyzed by the enzyme nitrate reductase (EC 1.6.6.1). The nitrate reductase is normally found in the cytosol, and its activity is regulated by several internal and external signals such as light, ATP, and NADPH. The latter two produced during photosynthesis, and regulated by C and N metabolites as sucrose and glutamine (Nunes-Nesi et al., 2010). Overall, the enzyme reaches the maximum activity at the

maximum leaf expansion (Marschner, 2012).

At 60 days after planting, untreated plants presented NO^{2-} values higher than those observed at 50 days after planting, indicating that the second sampling was the stage of the highest activity of the enzyme, which was increased by applications of fermented broth at the concentration of 0.357 mL L^{-1} (Figure 5).

The amino acid L-glutamic plays a key role in the efficiency of the nitrogen metabolism. It is the first compound produced in the assimilation of this element, forming the amides glutamine and asparagine, and from these, the transport N to the different organs of the plant, also for the chlorophyll and amino acids synthesis (Ford & Lea, 2007).

Wherefore, the alterations in the nitrate reductase activity related to the fermented broth applications as the same way that were found for chlorophyll and free amino acid, link the enzyme activity to photosynthetic activity, and to the senescence delay. Thus, the increase in the total soluble protein content in the potato leaves due to the increases in nitrate reductase activity is expected and confirmed, especially at 60 days after planting, notedly at 0.325 mL L^{-1}.

Conclusions

The applications of the fermented broth on organically grown potato promoted remarkable yield gains and alterations in chlorophylls, carotenoids, nitrate reductase activity, delaying the senescence and enhancing the contents of free amino acids and soluble proteins in leaves, allowing to infer that the L-glutamic acid is a natural active compound in the fermented broth, showing up as an efficient natural tool to contribute for sustainability of potato production. It was concluded that the fermented broth could be classified as a biofertilizer due to its ability of improving potato yield by stimulating the nitrate reductase activity.

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