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Can be performance indexes used to select plant growth-promoting rhizobacteria?

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Abstract

The plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the rhizosphere or roots, resulting in a beneficial effect on plants. The evaluation of the PGPR effects is mostly based on growth biometric measurements, but there is little information about using the chlorophyll (Chl) fluorescence. We used rhizobacteria to treat canola seeds and evaluate their effects on leaf area, dry mass, and Chl fluorescence. In order to select PGPR, we suggest the JIP-test as a method to evaluate their effects using the performance indexes. The biometric measurements and JIP-test parameters allowed to group bacterial isolates into different groups. We observed that some PGPR increased the energy absorption efficiency/or flux. We conclude that the performance indexes can be used to differentiate the interaction of plants and PGPR and to select rhizobacteria with a potential for plant growth promotion.

Additional key words: *Brassica napus* L.; fluorescence transient; OJIP curve.

Introduction

Soil microorganisms are essential for the maintenance and sustainability of animal and plant communities because they affect many fundamental biological processes (Ambrosini *et al.* 2012). Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the rhizosphere or roots, resulting in a beneficial effect on plants (Abbasi *et al.* 2011). Some of the action mechanisms are: nitrogen fixation (Zhang *et al.* 1997, Vessey 2003, Ahemad and Khan 2012, Satapute *et al.* 2012), phosphate solubilization (Ramani 2011), synthesis of plant growth regulators (Ma *et al.* 2011, Walia *et al.* 2014), siderophore production (Yu *et al.* 2011, Noël *et al.* 2014), limitation of ethylene inhibitory responses through the enzyme 1-aminocyclopropane-1-carboxylate deaminase (Ahmad *et al.* 2013, Barnawal *et al.* 2013), and biocontrol agents against phytopathogens (Bhattachaeyya and Jha 2012).

The complex network of interactions occurring between plants and PGPR can affect plant growth and development directly or indirectly (Liu *et al.* 2013). PGPR stimulate plant growth through nutrient mobilization, improvement of soil structure, and production of growth regulators (Bulgarelli *et al.* 2013). Indirect mechanisms include antagonism against pathogens through competition with deleterious microorganisms for nutrients and synthesis of siderophores, antibiotics, and enzymes (Karakurt and Kotan 2011). The use of PGPR in sustainable systems can be a viable technology to increase plant production with lowering the use of chemical fertilizers and pesticides, with consequent decreases in risks for the environment and humans (Souza *et al.* 2013, Ahemad and Kibret 2014).

The effects of these microorganisms on plant development have been studied in several crops, and their benefits in specific biosystems have been reported (Mathesius and Watt 2011, Saharan and Nehra 2011). Evaluation of the PGPR effects on the plants is mostly based on biometric measurements of growth, such as plant height, number

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Abbreviations: ABS/RC – specific absorption flux per reaction center; cfu – colony forming units per mL; DM – dry mass; ET_0/RC – maximal specific flux for electron transport further than Q_A^-; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; $F_v/F_m = \varphi_{P0}$ – maximum photochemical efficiency of PSII; LA – leaf area; PGPR – plant growth-promoting rhizobacteria; PI_{ABS} – performance index; PI_{total} – total performance index; RC – reaction center; RE_0/RC – electron flux for reducing end electron acceptors at the PSI acceptor side per RC; TR_0/RC – specific trapping flux at t = 0; φ_{E0} – quantum yield for electron transport at t = 0; φ_{R0} – quantum yield for reduction of end electron acceptors at the PSI acceptor side.
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of leaves, leaf area, and biomass mass. The recent work showed an inappropriate use of fresh mass to report the effect of PGPR on crop plants (Huang *et al.* 2017). Moreover, biometric measurements are destructive and time-consuming, therefore not feasible for selection process.

Generally, the selection of microorganisms *in vivo* is somewhat complicated by the diversity and complexity of their interactions with plants. Thus, it is necessary to develop efficient strategies for selection, in order to reduce costs and to increase probabilities of selecting efficient organisms (Köhl *et al.* 2009, 2011). In this sense, some authors proposed the use of multiple *in vitro* traits to quantify plant growth promotion abilities and the potential of biocontrol (Agaras *et al.* 2015, Mota *et al.* 2017). However, to our knowledge, no nonbiometric *in vivo* criterion has been proposed for selecting PGPR so far.

JIP-test is a fast and efficient nonbiometric method which evaluates photosynthetic characteristics. The JIP-test parameters, which allow the analysis of Chl *a* fluorescence emission kinetics, describe the energy flow through the chloroplast electron transport chain. The Chl *a* fluorescence transients were recorded *in vivo* and analyzed according to the JIP-test leading to the calculation of a group of parameters, quantifying the PSII behavior. These parameters demonstrate how energy is absorbed and the efficiency of its use in the biophysical processes occurring in thylakoids (Stirbet and Govindjee 2012, Banks 2017); they can be grouped in: the nonphotochemical process, the absorption/flux energy, and the quantum yields. The analysis of Chl *a* fluorescence has become a highly efficient tool to investigate physiological events and to obtain detailed information on the structure and functioning of the photosynthetic apparatus (Dayan and Zaccaro 2012). The Chl *a* fluorescence techniques represent highly versatile tools appropriate for many applications, including plant physiology, biophysics or biochemistry; it is noninvasive, highly sensitive, and simple method. The multiparametric analysis of the transient fluorescence rise (OJIP) have been compared to a bar code for photosynthesis, and extensive attempts to simulate OJIP transients have been made.

The analysis of Chl *a* fluorescence can be easily used for the analysis of many samples of photosynthesizing organisms (Tsimilli-Michael *et al.* 2000, Murchie and Lawson 2013). In addition, the OJIP curve has been used in studies with physiological and biophysical or biochemical focus, as well as in applied research, to identify genotypes that display more efficient use of the energy absorbed by the photosystems (Baker and Rosenqvist 2004), and to study the interactions between nitrogen-fixing bacteria and mycorrhizal fungi in alfalfa (*Medicago sativa* L.) (Tsimilli-Michael *et al.* 2000).

The aim of the present study was to evaluate Chl *a* fluorescence as a key parameter to select PGPR. In this sense, we used rhizobacteria with *in vitro* and/or *in vivo* known skills to treat canola (*Brassica napus* L. var. *oleifera*) seeds. We compared biometric and photosynthetic measurements to validate the proposed selection criterion.

Materials and methods

Bacterial isolates: Seeds from the canola (*Brassica napus* L. var. *oleifera*), hybrid Hyola 433, were microbiolized with 96 bacterial isolates from the collection of the Laboratory of Plant Bacteriology of the Federal University of Pelotas, Brazil. These bacteria (DFs code) were isolated from soil and different species belonging to many botanic families including Brassicaceae. These bacteria had *in vitro* and/or *in vivo* known skills: 24 isolates showed biocontrol *in vivo* for at least one phytopathogen (bacteria, fungi or nematode) and/or growth promotion (common beans, fig, garlic, kale, onion, peach, rice, and tomato). The others produced *in vitro* antimicrobial compounds and enzymes related to biocontrol and/or growth promotion: seven isolates had all eight abilities evaluated; 35 isolates did not produce only one of the compounds (usually chitinases); 25 isolates showed six traits (also usually did not produce chitinases); and to contrast, the other two isolates produced only one or two compounds (Zanatta *et al.* 2007, Corrêa *et al.* 2014, Naue *et al.* 2014, Mota *et al.* 2017, Souza Júnior *et al.* 2017).

Seeds microbiolization: Bacterial suspensions were prepared by individually growing the rhizobacteria in '523' medium (Kado and Heskett 1970). After 24 h of growth, the bacterial colonies were suspended in sterile saline solution (0.85% NaCl), and their concentrations were adjusted using a spectrophotometer (*Ultrospec 2100 pro*, *Biochrom Ltd.*, USA) to absorbance (A_{540}) = 0.5, corresponding to approximately 10^8 colony forming units per mL [cfu mL^{-1}]. Seeds were immersed in the suspensions of each rhizobacteria for 4 h at 25 ± 1°C under constant stirring. For the control treatment, seeds were immersed in a sterile saline solution.

Sowing and growth conditions: Following microbiolization, six seeds from each treatment were placed in 0.5-L plastic pots containing a nonsterile soil:sand mixture (2:1). The pots were watered daily as needed to maintain a high moisture level of the substrate. Following seedling emergence, thinning was performed, leaving one plant per pot. The experiment was performed in a greenhouse at an 11.4°C mean temperature, irradiance of 250 μmol(photon) m^{-2} s^{-1} and 7.0 h of average insolation per day.

Chl *a* fluorescence transient: The polyphasic Chl *a* fluorescence transient was measured 55 d after sowing (DAS), when the plants were at the rosette stage with three to four fully expanded leaves. The measurements were performed using a portable fluorimeter (*Handy PEA*, *Hansatech Instruments*, King's Lynn, Norfolk, UK) during the morning, between 8 and 10 h, on the first fully expanded young leaves, which were previously dark-adapted for 30 min. Fluorescence emission was induced by applying a saturating light pulse of 3,000 μmol(photon) m^{-2} s^{-1}. The fluorescence signal was recorded for 1 s. Fluorescence values at 50 μs (F_0, initial fluorescence), 100 μs, 300 μs, 2 ms (F_J), and 30 ms (F_I), as well as F_m

(maximum fluorescence), were used to calculate the JIP-test parameters (Strasser and Strasser 1995, Tsimilli-Michael *et al.* 2000).

Biometric measurements: Immediately following the Chl *a* fluorescence measurement, the plants were harvested to determine the biometric parameters. The leaf area was measured using a leaf-area meter (*LI-3100*, *Li-Cor Inc.*, Lincoln, NE). The shoot dry mass was measured after the plant material was dried in a forced-circulation oven at 70°C.

Experimental design and statistical analysis: The experimental design was completely randomized, with five replicates per treatment, and experimental units consisted of one pot containing one plant. The data (biometric measurements and fluorescence parameters) were subjected to analysis of variance (*ANOVA*) using the *F* test, and the means were compared using the *Scott Knott*'s test with a 5% significance level, and presented in frequency histogram and scatter plot with *Pearson*'s coefficient and probability.

Results

Biometric measurements: The plant leaf area (LA) is shown in Fig. 1*A*. The treatments with bacterial isolates were statistically grouped into three groups: (*1*) Group I was composed of three isolates [DFs144 (11), DFs185 (16), and DFs628 (42)] with higher leaf area values than that of the remaining treatments, which were approximately 140, 120, and 90% higher than the values of the control plants (0.0016 m^2 per plant), respectively. (*2*) Group II was composed of 28 isolates with leaf areas 32–74% higher than the control treatment. (*3*) Group III contained the remaining isolates, which were not different from the control (Fig.1*B*). The shoot dry mass (DM) of the control plants was 0.104 g(DM) per plant (Fig. 1*C*). Three groups were established: (*1*) Group I was composed of isolates DFs144 (11) and DFs185 (16), which had the shoot dry mass 140 and 110% higher than the control treatment, respectively; (*2*) Group II was composed of 32 rhizobacteria presenting dry mass values 11–37% higher than the control treatment; and (*3*) Group III contained the remaining rhizobacteria, which were not significantly different from the control (Fig. 1*D*).

Chl *a* fluorescence analysis and JIP-test: We plotted the fluorescence intensity on a logarithmic time scale and the fast fluorescence kinetics of Chl *a* obtained for all the treatments presented the characteristic polyphasic OJIP transient (data not shown), indicating that all the samples were photosynthetically active (Yusuf *et al.* 2010). The JIP-test, that is a multiparametric analysis of the fast fluorescence rise (OJIP), developed by Strasser and Strasser (1995) (for a detailed explanation of the JIP-test *see* Tsimilli-Michael *et al.* 2000, Stirbet and Govindjee 2012), was used to translate the fluorescence intensity to numeric parameters that explain the energy in photosynthetic electron transport chain (ETC).

We observed that some PGPR increased the efficiency of the energy absorption and/or flux (Fig. 2), when compared with the control. The bacterial treatments were statistically grouped into three groups. Approximately 40% of the rhizobacteria tested resulted in significantly increased specific absorption flux per reaction center (RC) (ABS/RC) (Fig. 2*A*). Group I was formed by nine isolates: DFs96 (5), DFs119 (9), DFs149 (12), DFs567 (37), DFs622 (41), DFs1615 (79), DFs2121 (89), DFs2162 (92), and DFs2282 (95), that produced ABS/RC values significantly higher than the remaining rhizobacteria (9–19% higher than the control). In addition, Group II included 32 other isolates significantly higher than the control group (Group III). A total of 34% of the tested isolates constituted a statistic group with higher specific trapping flux at time zero (TR_0/RC) when compared to the control without rhizobacteria (Fig. 2*B*). This effect was more pronounced for DFs96 (5), DFs119 (9), DFs149 (12), DFs622 (41), and DFs1615 (79), which resulted in 9–16% increase in TR_0/RC.

Concerning the maximal specific flux for electron transport further than Q_A^- (ET_0/RC), 21 of the tested rhizo-bacteria resulted in increases higher than 10% compared to the control treatment (Fig. 2*C*). This effect was more pronounced for isolates DFs149 (12), DFs622 (41), DFs1625 (80), DFs1810 (82), and DFs2162 (92), which produced a 20% increase in ET_0/RC, which was not yet significant. In 30 rhizobacteria, the electron flux for reducing end electron acceptors at the PSI acceptor side per RC (RE_0/RC) increased by 10% compared to the control treatment (Fig. 2*D*). This effect was more pronounced for isolates DFs149 (12) and DFs622 (41), which presented a 20% nonsignificant increase in RE_0/RC.

The ABS/RC and TR_0/RC data of the control treatment were inserted in groups within the greater cumulative frequency (Group III and II, respectively) (Fig. 2*A*,*B*). In contrast, the ET_0/RC and RE_0/RC data were added in the lower frequency range (Fig. 2*C*,*D*).

The control plants presented maximum quantum yield for primary photochemistry (at t = 0) ($\varphi_{P0} = F_v/F_m$), nearly as the mean value for all isolates (0.796) (Fig. 2*E*). The quantum yield for electron transport (at t = 0) (φ_{E0}) and quantum yield for reduction of end electron acceptors at the PSI acceptor side (φ_{R0}) from the control plants showed lower values compared to most of the other treatments. For φ_{E0}, only eight rhizobacteria showed the increase between 10–15% compared to the control treatment (Fig. 2*F*), and for φ_{R0}, 18 rhizobacteria exhibited values approximately 10–13% higher than that of the control (Fig. 2*G*). In general, rhizobacteria showed lesser effect on quantum yields, and no differences between treatments were observed for φ_{P0}, φ_{E0}, and φ_{R0}.

The performance indexes (PI_{ABS} – performance index, PI_{total} – total performance index) (Fig. 3) showed values within a greater range than that of the photosynthetic parameters and the treatments, and both indexes were grouped in two groups: Group I – statistically higher performance index than that of the control without bacteria, and the Group II – similar to the control. Higher portion of the isolates (65%) provoked increase in PI_{ABS} when compared with the value of the control without bacteria (PI_{ABS} = 2.02, control)

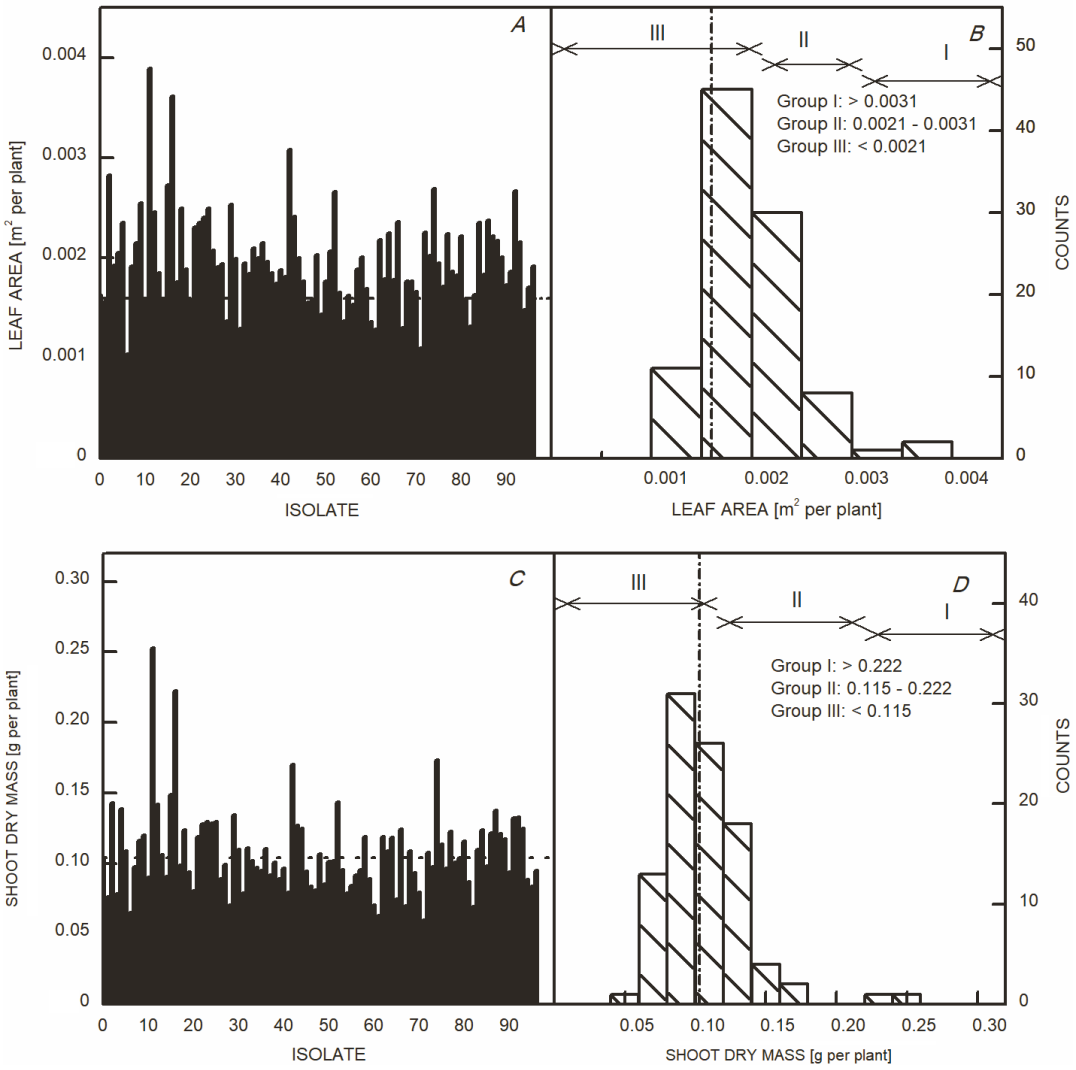

Fig. 1. Leaf area (*A*), frequency counts histogram of the leaf area (*B*), shoot dry mass (*C*), and frequency counts histogram of the shoot dry mass (*D*) of the canola plants originated from seeds microbiolized with different bacterial isolates obtained at 55 d after sowing. *Horizontal* and *vertical dot lines* indicate the value of the control plants. Group number represents the result of the *Scott-Knot*'s grouping test, and the number is the limit value for each group (total counts = 97).

(Fig. 3*B*), and 55% of the isolates raised PI_{total} when compared with control (PI_{total} = 3.04, control) (Fig. 3*D*). Bacterial isolates DFs185 (16), DFs320 (19), DFs359 (24), DFs439 (30), DFs465 (33), DFs582 (38), DFs658 (44), DFs1258 (63), and DFs2108 (88) showed the greatest increases in PI values, varying between 28 and 37% for PI_{ABS} and between 30 and 44% for PI_{total}.

When we associated the biometric parameters (LA and DM) with the performance indexes (PI_{ABS} and PI_{total}), we observed a positive *Pearson*'s correlation coefficient (r) at $p<0.01$ (Fig. 4). As the correlation is a statistical method used to assess a possible linear association between two continuous variables and is measured by the correlation coefficient, which represents the strength of the putative linear association between the variables in question, we can assume that there is a positive linear relationship between biometric parameters and performance index.

Discussion

Seed microbiolization with rhizobacteria results in complex interactions between these microorganisms and their hosts. These associations can interfere positively or negatively with plant growth. The effects are generally measured using biometric parameters. The PGPR's effects on our biometric parameters had a big range of values. We observed positive and negative effects; furthermore, the percentage of isolates with beneficial impacts was high. In the current experiment, the isolates DFs144 (*Bacillus thuringiensis*), DFs185 (*Pseudomonas synxantha*), and DFs628 (*Bacillus subtilis*) resulted in the greatest increases in the plant growth biometric parameters. Plant growth-promoting responses resulting from seed or soil inoculation with *Bacillus* and *Pseudomonas* rhizobacteria have been observed in several species. Gupta *et al.* (2012) observed

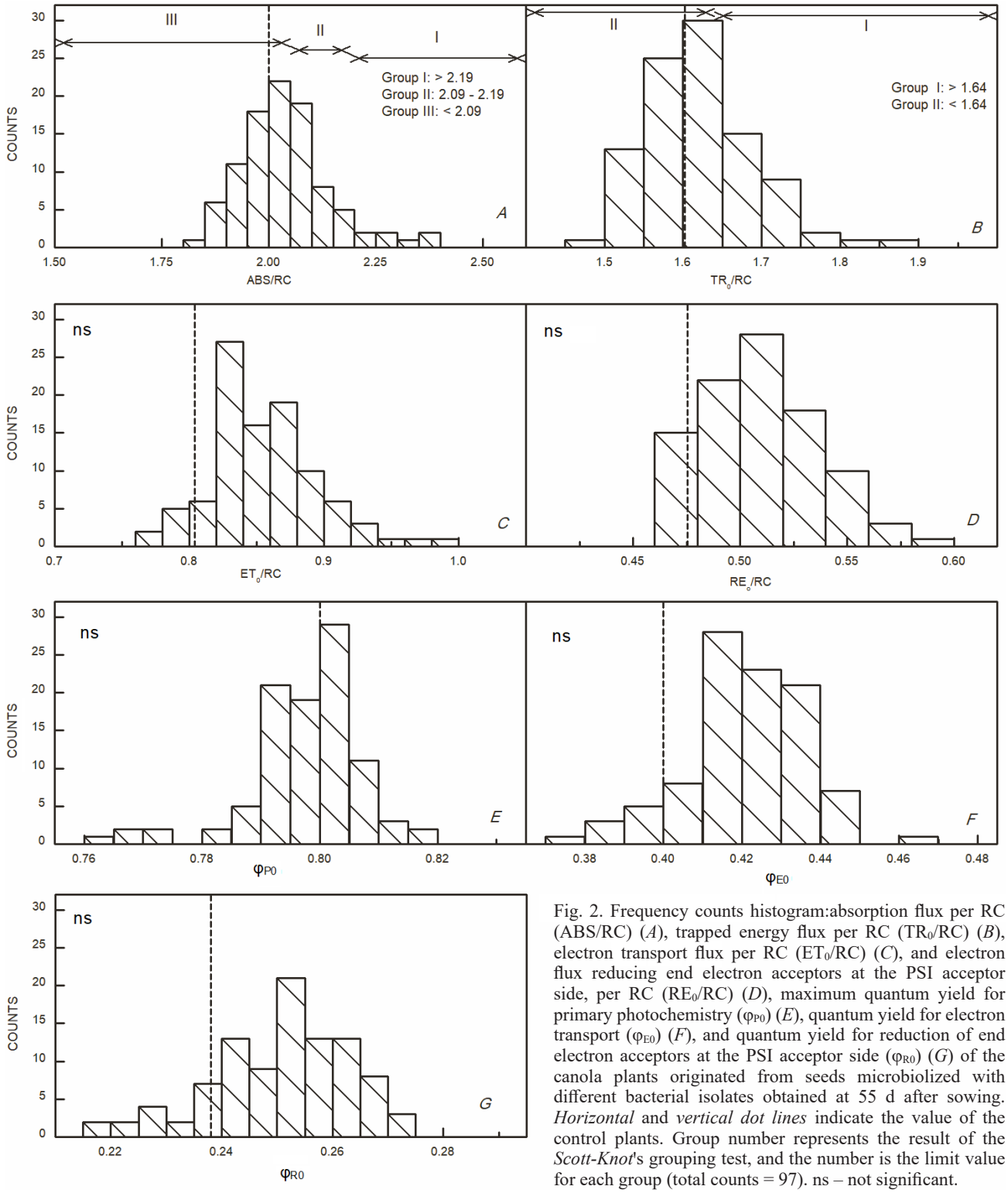

Fig. 2. Frequency counts histogram:absorption flux per RC (ABS/RC) (*A*), trapped energy flux per RC (TR_0/RC) (*B*), electron transport flux per RC (ET_0/RC) (*C*), and electron flux reducing end electron acceptors at the PSI acceptor side, per RC (RE_0/RC) (*D*), maximum quantum yield for primary photochemistry (φ_{P0}) (*E*), quantum yield for electron transport (φ_{E0}) (*F*), and quantum yield for reduction of end electron acceptors at the PSI acceptor side (φ_{R0}) (*G*) of the canola plants originated from seeds microbiolized with different bacterial isolates obtained at 55 d after sowing. *Horizontal* and *vertical dot lines* indicate the value of the control plants. Group number represents the result of the *Scott-Knot*'s grouping test, and the number is the limit value for each group (total counts = 97). ns – not significant.

increases in biometric parameters, such as the root length, leaf number, and dry mass, of *Aloe barbadensis* inoculated with *P. synxantha*. Microbiolization of corn seeds with *Bacillus thuringiensis* and *Bacillus subtilis* resulted in enhanced total dry mass, shoot height, and root length (Vardharajula *et al.* 2011). The authors also noted that the increase in plant growth was observed when plants were under water stress. Liu *et al.* (2013) reported that the inoculation of *Fraxinus americana* with *B. subtilis*, associated with fertilization, promoted plant growth and nutrient uptake.

Previously, Tsimilli-Michael *et al.* (2000) proposed

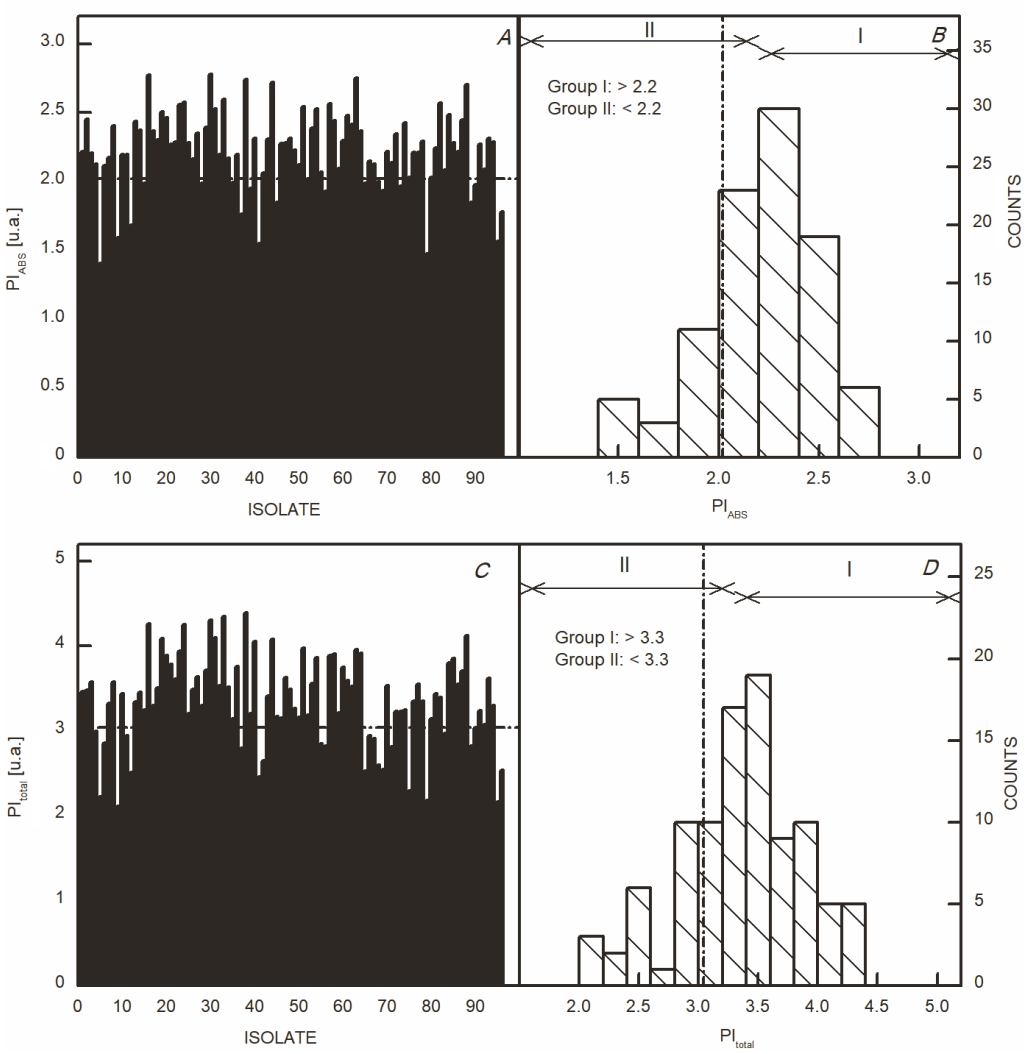

Fig. 3. Performance index (PI_{ABS}) (*A*), frequency counts histogram of the PI_{ABS} (*B*), and total performance index (PI_{total}) (*C*), and frequency counts histogram of the PI_{total} (*D*) of the canola plants originated from seeds microbiolized with different bacterial isolates obtained at 55 d after sowing. *Horizontal* and *vertical dot lines* indicate the value of the control plants. Group number represents the result of the *Scott-Knot*'s grouping test, and the number is the limit value for each group.

the use of Chl *a* fluorescence to evaluate synergistic and antagonistic effects between microorganisms and plants, microbial activity in the field, and comparison of the whole ecosystems or individuals within an ecosystem. The analysis of Chl *a* fluorescence facilitates evaluation of plant photosynthetic characteristics (Strasser *et al*. 2004), as it enables the study of the PSII behavior/activity and can be used to detect positive plant responses to the PGPR action.

We observed no differences for maximum quantum yield for primary photochemistry (φ_{P0}) in plants with or without bacteria treatments. However, many studies use the Chl *a* fluorescence to report the F_v/F_m (φ_{P0}) as the parameter, which can be used to detect changes in the photosynthetic apparatus (Kalaji *et al.* 2017). The maximum quantum yield for primary photochemistry is not always sensitive enough to detect differences between treatments or initial variations caused by abiotic stresses (Redillas *et al.* 2011, Koler *et al.* 2013, Kalaji *et al.* 2017). In the present study, it was not possible to differentiate treatments using φ_{P0}. A similar result was also reported by Zhang *et al.* (1997) for biotic stress; they observed that φ_{P0} was not changed in soybean plants inoculated with *Aeromonas*, *Pseudomonas*, and *Serratia* rhizobacteria.

The changes in the JIP-test parameters observed in the present study reflect possible interactions between the rhizobacteria and the canola plants. However, due to the complexity of the parameters (Tsimilli-Michael *et al.* 2000, Strasser *et al.* 2004), it is difficult to describe the behavior of the PGPR association with the host canola plants using a single parameter. Recently, different methods or parameters for data interpretation have been suggested, but the best way to properly evaluate the performance of plants is to combine many parameters in one multiparametric expression (Stirbet *et al.* 2018). In this sense, the performance index (PI_{ABS}) combines

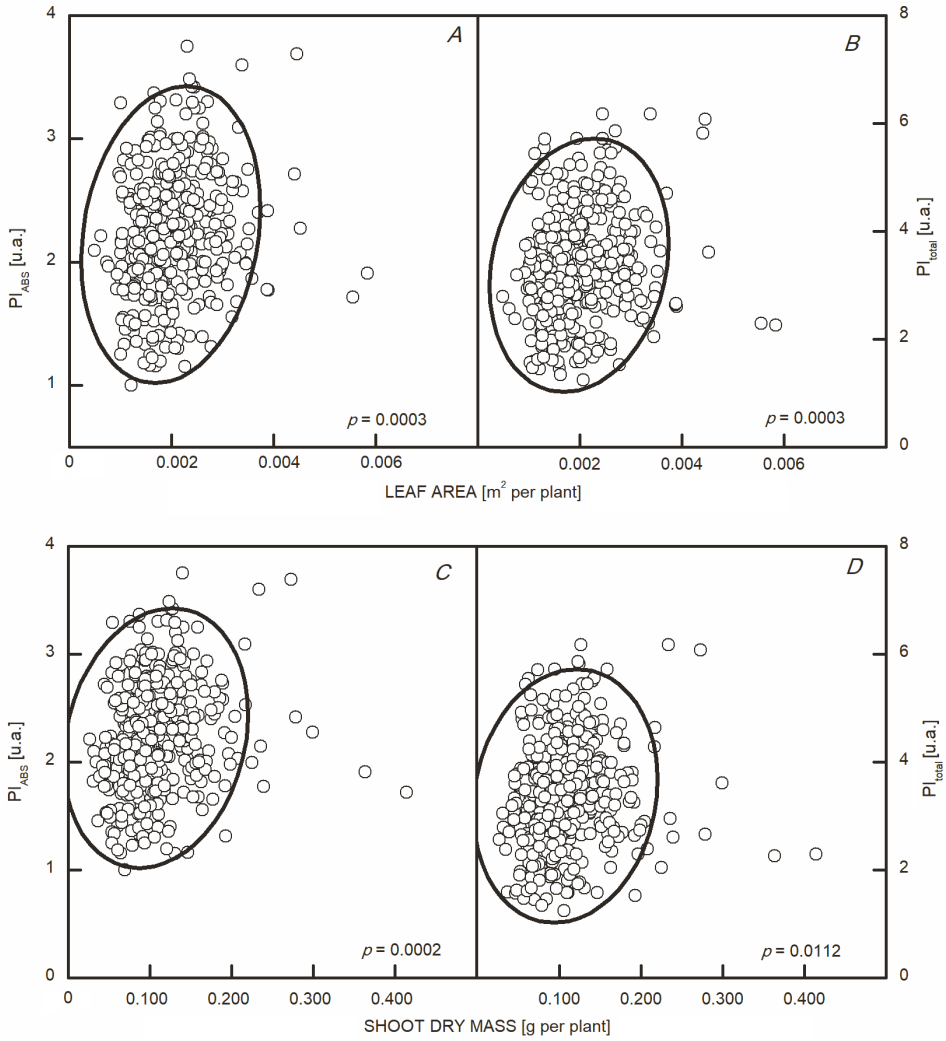

Fig. 4. Scatterplot between leaf area and PI_{ABS} (*A*), leaf area and PI_{total} (*B*), shoot dry mass and PI_{ABS} (*C*), and shoot dry mass and PI_{total} (*D*) of the canola plants originated from seeds microbiolized with different bacterial isolates obtained at 55 days after sowing. The number insert in each panel represents the probability (*p*) of the *Pearson*'s correlation.

three partial components: the density of fully active RC (RC/ABS), the maximal energy flux reaching the RC of PSII [$\varphi_{P0}/(1 - \varphi_{P0})$], and electron transport at the onset of illumination [$\Psi_{E0}/(1 - \Psi_{E0})$]. The total performance index (PI_{total}) adds an extra component to PI_{ABS}, which describes the reactions of oxidation and reduction occurring on the electron acceptor side of PSI [$\delta_{R0}/(1 - \delta_{R0})$]. Variations in these two parameters allow the detection of changes in the electron transport chain more easily than a single parameter.

Seed microbiolization with most of the tested bacterial isolates increased the performance indexes of the host plants. In 86% of the rhizobacteria, the results showed the plant growth promotion. Increase in leaf area and shoot dry mass occurred together with increases in PI_{ABS}, PI_{total} or both. This result shows that PI_{ABS} and PI_{total} can be used to differentiate the plant photosynthetic performance affected by different microbiolization treatments and to identify rhizobacteria that result in greater accumulation of plant biomass.

The results obtained in the present study show that the analysis of Chl *a* fluorescence is an important tool to study the photosynthetic activity in plants originated from seeds microbiolized with PGPR, with the advantage of being a nondestructive and quick method. The analysis of the fluorescence transient using the JIP-test indicated that PI_{ABS} and PI_{total} can be used to detect differences in plant vitality (Tsimilli-Michael *et al.* 2000, Strasser *et al.* 2004, Stirbet *et al.* 2018), in terms of photosynthetic energy conservation resulting from microbiolization, even before biometric differences due to the interaction between rhizobacteria and the host plant became evident.

We also suggest that the performance indexes can be used as a screening tool for comparing the efficiency of plants inoculation with different microorganisms and selecting the better ones. PI_{ABS} was also successfully used to detect synergic and antagonistic effects of the combination between the bacteria *Azospirillum brasilense*

and *Rhizobium meliloti* and the mycorrhizal fungus *Glomus fasciculatum* in alfalfa (Tsimilli-Michael *et al.* 2000).

Conclusion: Biometric measurements showed the growth promotion in canola plants due to the seed microbiolization with PGPR. The chlorophyll *a* fluorescence transient can be used to evaluate the photosynthetic responses of plants originated from seeds microbiolized with rhizobacteria. The photosynthesis performance indexes (PI_{ABS} and PI_{total}) can be used to differentiate the PGPR effects on plants and can be also used to select rhizobacteria with potential for plant growth promotion.

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