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EXPERIMENTAL ARTICLES

A Plant Growth-Promoting Rhizobacteria (PGPR) Mixture Does Not Display Synergistic Effects, Likely by Biofilm But Not Growth Inhibition[1]

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Abstract—Combination of different PGPR strains with complementary characteristics as a mixture to reduce possible instability under fluctuating environment has been considered practical. However, PGPR mixtures do not always play synergistic roles in growth promotion or biological control as reflected in our previous findings and other publications. In this work, we accidentally discovered that a mixture containing two well compatible PGPR strains, *Bacillus pumilus* WP8 and *Erwinia persicinus* RA2, did not synergize in growth promotion or biological control of tomato bacterial wilt under field conditions. Considering the importance of PGPR biofilm formation in growth promotion and biocontrol activities, we hypothesized that this phenomenon may be related to inhibition of biofilm formation. In vitro experiments showed that biofilm-formation ability of WP8 was inhibited by both RA2 cells and filtered supernatants collected from RA2 cultures at 12 h (RA2–12) rather than 48 h (RA2–48), even at high-temperatures (within 100°C). An in vivo experiment derived from crystal violet staining yielded similar results. Using liquid chromatography-mass spectrometry (LC–MS), we compared primary and secondary metabolites of RA2 (namely RA2–12 and RA2–48) and found D-glutamine, abundant in RA2–12, as the putative inhibitory factor. Trace amounts of jasmonic acid together with viscous extracellular polysaccharides in RA2–48 likely promoted the rescue of robust biofilm formation. This work suggests that inhibition of biofilm formation should be considered in PGPR mixture development.

Keywords: plant growth-promoting rhizobacteria (PGPR), synergistic effect, biofilm formation, inhibition, mixture

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Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can grow in, on, or around plant root tissues, stimulating plant growth directly and/or indirectly [1, 2]. PGPR combination is known to be a highly promising approach to achieving green and sustainable agriculture goals by reducing chemical inputs in agricultural production. Over the past few decades, many PGPR strains belonging to various genera, such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* have been isolated from different environments, and some of them developed and successfully used as commercial products [2, 3]. Nevertheless, the major barrier to large-scale application of these agents is the instability of PGPR since good results obtained in vitro or in a certain environment do not always dependably reproduce under field or other unusual conditions [4], due to different nutrient availability and other environmental stimuli, such as major and minor minerals, type of carbon source and supply, pH, temperature, heavy metals in soils and other parameters [5–8]. Thus, considering the diversity of PGPR mechanisms of action, combination of different PGPR strains with complementary characteristics as a mixture to reduce possible instability under fluctuating environment has been considered practical [1, 9, 10].

Unfortunately, PGPR mixtures do not always display synergistic effects in growth promotion or biological control. For instance, anthracnose disease incidence of long cayenne pepper inoculated with different PGPR mixtures did not significantly decrease compared with treatment by a single PGPR strain in the rainy season [10]. In addition, a mixture of three PGPR strains reduced disease incidence and promoted growth to a level equivalent to two strain mixtures [11]. A clue to explain such phenomena might be found in the prerequisite for successful development of PGPR mixtures that strains must be compatible [12]. In other words, the reported PGPR mixtures which did not exhibit synergistic effects may have not been compatible in vitro. We accidentally found that some

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PGPR strains doubtlessly compatible still did not exhibit synergistic effects. Considering that PGPR must establish and maintain critical population densities in rhizosphere to exert growth-promoting effects [13–15], in addition to the fact that many PGPR achieve growth-promoting or biocontrol effects by forming biofilms [16, 17], we hypothesized that PGPR not synergizing may be related to inhibition of biofilm formation. Therefore, we suggested that inhibition of biofilm formation should be considered in designing PGPR mixtures. The present study was designed to test the hypothesis with two PGPR strains in both in vitro and in vivo experiments.

MATERIALS AND METHODS

Microbial strains and inoculum preparation. *B. pumilus* WP8 and *Erwinia persicinus* RA2 were isolated from rhizosphere soil of wheat (*Triticum aestivum* L.) and rape (*Brassica chinensis* L.) in the experimental plot of Yangzhou University, China [3]. The two PGPR strains were separately grown at 28°C for 48 h in nutrient broth (NB) on a rotary shaker (180 rpm). Cells were harvested by centrifugation (5000 *g* for 10 min) and bacterial pellets resuspended in sterile distilled water (SDW) at 10^9 colony-forming units (CFU) mL^{-1} as determined by plate count on nutrient agar (NA). The phytopathogen *Ralstonia solanacearum* Rs 1115, donated by Prof. Shen (Nanjing Agricultural University) causes tomato bacterial wilt (TBW). Rs 1115 cell suspension of was obtained as described for PGPR strains.

Preparation of RA2 supernatants. RA2 supernatants were collected after 12 (RA2–12) and 48 h (RA2–48) incubation by centrifugation at 5000 *g* for 10 min followed by filtration with 0.22 μm and stored at 4°C until use.

In vitro test for PGPR strain compatibility. The plate confrontation culture method was adopted to assess the compatibility of PGPR strains. Briefly, a total of 50 μL WP8 cell suspension was spread on NA plate, and RA2 streaked onto the center. After 5 days of culture, plates were imaged using a Canon digital IXUS 15 camera.

Biofilm assay. The biofilm assay on polystyrene was performed by the method described by O'Toole and Kolter [18]. Briefly, WP8 or RA2 were cultured overnight in tryptic soy broth (TSB) glucose arid the cultures diluted 250-fold into each well of flat-bottom 96-well polystyrene plates. After incubation at 32°C for 48 h, cells were washed three times with SDW, air dried, and stained with 0.4% crystal violet for 15 min. Plates were washed four times with SDW to remove unbound crystal violet and dried for 2 h at 32°C. Then, 95% (v/v) ethanol was added to each well and incubated for 10 min on a shaker to release the dye from the biofilms; absorbance was read at 570 nm on an enzyme-linked immunosorbent assay plate reader.

For temperature tolerance of culture supernatants of RA2, D-glutamine and jasmonic acid (JA) in WP8 biofilm formation, samples were heated at 40, 70, 100 and 121°C for 15 min, respectively, and added into each well at the final concentration of 20%, 7 mM, and 15 μM, for RA2–12, D-glutamine, and JA, respectively. Afterward, the procedures described above were used for inoculation, incubation and assessment. To assess RA2 cell effects on biofilm WP8 formation, equal amounts of RA2 and WP8 cultures were co-inoculated into wells.

Growth assessment. To determine the effect of RA2 supernatants on WP8 growth, 200 mL TSB containing 0 (control), 5, 10 and 20% RA2–12 or RA2–48 were loaded in flasks. Upon inoculation with WP8, absorbance at 600 nm was measured every 4 h to evaluate growth.

In vivo effect of RA2 supernatants on biofilm formation by WP8 on tomato root surface. Tomato seeds (Jingdan No. 1), sterilized with 70% ethanol for 30 s and 4% NaClO solution for 20 min, were germinated in 9 cm sterile petri dishes covered with moist filter paper for 3 days and subsequently immersed in WP8 cell suspensions containing fresh TSB, RA2–12, RA2–48 and equivalent RA2 cell suspension, respectively, for 20 min. Then, the cultures were air dried under a laminar flow hood. The treated seeds were transplanted onto half-strength MS medium in glass tubes (25 × 250 mm) and allowed to grow for 12 days at 25°C. The resulting seedlings were carefully extracted out of the medium and rinsed with SDW and air dried. Roots were stained with 0.4% crystal violet for 15 min, washed four times with SDW to remove unbound crystal violet dye, and dried for 2 h at 32°C. The stained roots were immersed in 95% ethanol for 10 min followed by absorbance measurement at 570 nm. Data were presented as OD_{570nm} per centimeter: of root length in order to eliminate variations of root length in different treatment.

Field experiment for biological control of TBW by PGPR, alone or as mixture. A field experiment was carried out in randomized block designed with three replications per treatment at Yancheng Teachers University (Yancheng, China) from April to May 2012. The average monthly temperature and rainfall during the period were 16.2°C and 68.2 mm, respectively, based on data provided by the Bureau of Meteorology. Characteristics of sandy loam soil were: pH 7.6 (1 : 1 soil : water); organic matter, 3.83 g kg^{-1}; total nitrogen (N), 1.49 g kg^{-1}; available N, 19.44 mg kg^{-1}; phosphorus (P), 4.62 mg kg^{-1}; and potassium (K), 2.25 mg kg^{-1}. Tomato (*Lycopersicon esculentum* Mill.) seeds were pre-soaked with PGPR inoculum for 20 min before sowing. Each replicate plot was spread with 500 mL of Rs 1115 inoculum to moisten the topsoil, and controls were treated with equivalent volumes of tap water. Thirty seeds pre-inoculated with PGPR were sown in plots (1 × 2 m) separated by 1 m, and no other inoculation or fertilizer application was made

Table 1. Effects of *Bacillus pumilus* WP8 and *Erwinia persicinus* RA2 (alone or mixture) on biological control of TBW under field conditions

Treatments	Emergence rate at 17 DAS, %	Plant height, cm	Root length, cm	Stem diameter, mm	Aboveground dry weight, mg	Root dry weight, mg	Biocontrol efficiency (%)
Control	41.67 ± 2.89 c	7.10 ± 0.91 c	4.81 ± 0.86 b	3.20 ± 0.17 b	20.67 ± 4.73 c	4.17 ± 0.76 b	–
R.s	15.43 ± 2.21 e	13.99 ± 1.17 b	5.45 ± 0.34 ab	3.13 ± 0.45 b	23.67 ± 4.73 c	3.63 ± 1.10 b	–
WP8 + R.s	73.53 ± 2.20 a	29.06 ± 3.26 a	6.73 ± 0.85 a	4.47 ± 0.25 a	50.67 ± 4.04 a	11.33 ± 2.08 a	70.59±2.45a
RA2 + R.s	49.17 ± 6.29 b	17.12 ± 1.84 b	4.50 ± 0.89 b	2.77 ± 0.12 b	25.00 ± 4.00 c	4.00 ±1.00 b	43.52 ±6.99b
WP8 + RA2 + R.s	28.52 ± 2.57 d	14.60 ± 2.07 b	5.60 ± 1.35 ab	3.30 ± 0.40 b	32.67 ± 2.08 b	5.67 ± 0.58 b	20.57±2.85c

R.s represents the pathogen treatment caused by *Ralstonia solanacearum* Rs 1115. Emergence rate of tomato was assessed at 17 days after sowing (DAS), growth indicators including plant height, root length, stem diameter, aboveground and root dry weight were obtained by routine methods at 40 DAS. Values are means of three independent replicates ±SE. Different letters mean significant differences (Duncan's multiple range test, at $p < 0.05$) among treatments.

during the growth period. Main plot treatments were: (1) Control; (2) Pathogen control; (3) WP8; (4) RA2; (5) WP8 + RA2. Emergence rate of tomato was investigated 17 days after sowing (DAS), and growth indicators, including plant height, root length, stem diameter, aboveground and root dry weight were obtained by routine methods at 40 DAS. Biocontrol efficiency was also investigated at 40 DAS before sampling, and the data were computed according to the following formula:

$$\text{Biocontrol efficiency (\%)} = \frac{\text{(number of infected plant of pathogen control} - \text{number of infected plant of treatment)}}{\text{Number of infected plant of pathogen control}} \times 100\%.$$

Another field experiment without additional pathogen was conducted; to confirm the observed effect of PGPR (alone or mixture) on tomato growth from September to October 2012. The average monthly temperature and rainfall during this period were 19.2°C and 86.3 mm, respectively, based on data provided by the Bureau of Meteorology. Treatment and management were carried out as described above, except that no pathogen was added.

Comparison of RA2–12 and RA2–48 by liquid chromatography–mass spectrometry (LC–MS). To determine the compounds in RA2–12 responsible for biofilm inhibition, RA2–12, RA2–48, and blank control were analyzed by LC–MS. Protein were precipitated by mixing 200 μL of each sample with 600 μL methyl alcohol. After centrifugation at 13400 × g for 5 min, supernatants were used for analysis on an ACQUITY UPLC/Xevo G2 Q TOF (Waters Corporation, Milford, MA, USA) HPLC and MS instrument. Samples (5 μL) were injected into and separated on a UPLC BEH C18 column (Waters, 1.7 μm, 2.1 × 50 mm) at flow rate of 0.3 mL/min. Mass values corresponding to different peaks were searched in the METLIN database (http://metlin.scripps.edu/metabo_search.php) by selecting $[M + H]^+$ ions for automated identification with a mass tolerance of 0.05 Da.

RESULTS

Biological control of tomato bacterial wilt by PGPR alone or mixture. A field experiment (Table 1) showed that seedling emergence rates were enhanced by 376.54 and 218.66% by WP8 and RA2, respectively, as compared with pathogen control (R.s), while treatment with the mixture only enhanced this value by 84.83%, significantly lower than what observed for control plants. Our data showed asimilar results in other growth parameters such as plant height, root length and stem diameter among RA2, R.s and mixture treatments. Interestingly, WP8 exerted the most significant effect on seedling growth in comparison with other treatments. Indeed, the highest biocontrol efficiency appeared in WP8 treatment group (70.59%), followed by RA2 (43.52%) and mixture (20.57%), treatments, and the difference between these groups was statistically significant.

In vitro biocompatibility of WP8 and RA2. To determine if growth inhibition was the reason for above phenomenon, the plate confrontation culture method was adopted to assess the compatibility of these two PGPR strains. As shown in Fig. 1, WP8 and RA2 displayed a good compatibility.

Mutual inhibition of biofilm formation in PGPR Mixture. The quantitative biofilm assay data demonstrated that WP8 had a great ability of biofilm formation while RA2 scarcely form biofilm in vitro (Fig. 2a). Biofilm formation was more pronounsed with increasing glucose concentrations. We then analyzed the

effect of RA2 supernatants on WP8 growth and biofilm formation ability. As shown in Fig. 2b, addition of RA2 supernatants did not inhibit WP8 growth. However, supernatants collected at different times from of RA2 cultures (RA2–12 and RA2–48, collected at 12 and 48 h, respectively), exerted distinct effects on biofilm formation in WP8 (Fig. 2c). Biofilm formation by WP8 was significantly inhibited by RA2–12 in a concentration-dependent manner. Interestingly, RA2–48 displayed no inhibitory effects, and biofilm density decreased dramatically (by 60.0%) in co-culture treatment (addition of RA2 cells) in comparison with controls.

In vivo effect of RA2 supernatants on biofilm formation by WP8 on tomato root surface. As shown in Fig. 2d and Fig. 3, WP8 treatment showed similar effects observed after RA2–48 treatment, of which robust biofilm was obtained on tomato roots. However, addition of both RA2–12 and RA2 cells strongly inhibited the biofilm formation by WP8.

Effects of PGPR alone or mixture on tomato growth. A field experiment without additional pathogen was conducted to confirm the effect of PGPR, alone or mixture, on tomato growth (Table 2). WP8 treatment produced the most powerful impact on both emergence rate and seedling growth.

RA2 also played an important role in growth promotion, second to WP8. However, PGPR mixture still did not exert synergistic effects, and tomato growth was even inferior to both PGPR strains, used alone.

Chemical composition of RA2–12 and RA2–48. Using LC–MS technology, we compared the different metabolites in RA2 supernatants, namely RA2-12 and RA2–48. As shown in Fig. 4, D-glutamine, a putative metabolite in RA2–12, was more abundant in this sample than RA2–48 and controls. In RA2–48, another putative metabolite, jasmonic acid (JA) was especially conspicuous.

DISCUSSION

In previous studies, we have shown that WP8 and RA2 have a great potential as inoculum agents in promoting plant growth and biological control of TBW [19–21]. While assessing the effect of a combination of these two PGPR strains on biological control of TBW, we surprisingly found that the mixture did not exert the expected synergistic effect, although there was no incompatibility between WP8 and RA2 (Fig. 1). Considering that PGPR must establish and maintain critical population densities in rhizosphere to exert growth-promoting effects [13–15], in addition to the fact that many PGPR achieve growth-promoting effects by forming biofilms in the rhizosphere [17], we hypothesized that these PGPR not synergizing may be related to inhibition of biofilm formation. Compared with WP8. RA2 hardly formed biofilms in vitro (Fig. 2a), which may at least in part explain why growth-promoting ability of RA2 was inferior to that of WP8.

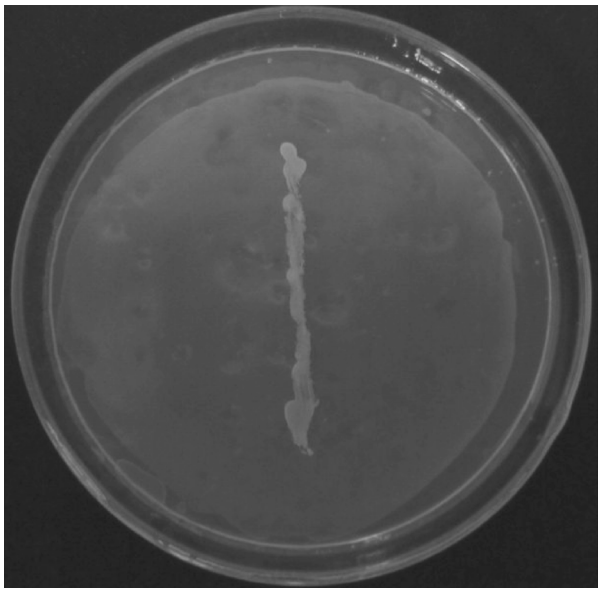

Fig. 1. Compatibility *B. pumilus* WP8 and *E. persicinus* RA2 (streaked one) determined by the plate confrontation culture method.

Using an in vitro method, we demonstrated that the biofilm-forming ability of WP8 was inhibited by both RA2–12 (concentration dependent) and RA2 cells, but not RA2–48 (Fig. 2c), suggesting that the inhibitory factors may be primary rather that second-

Table 2. Effects of *B. pumilus* WP8 and *E. persicinus* RA2 (alone or mixture) on tomato plant growth

Treatments	Emergence rate at 17 DAS, %	Plant height, cm	Root length, cm	Stem diameter, mm	Aboveground dry weight, mg	Root dry weight, mg
Control	56.81 ± 3.42 c	8.75 ± 0.69 c	5.24 ± 0.24 c	3.87 ± 0.16 b	21.76 ± 2.10 c	4.52 ± 0.28 d
WP8	83.68 ± 3.41 a	31.17 ± 6.18 a	6.99 ± 0.33 a	4.99 ± 0.24 a	62.14 ± 2.36 a	14.55 ± 0.91 a
RA2	63.22 ± 2.08 b	27.13 ± 1.56 a	6.20 ± 0.26 bc	3.47 ± 0.31 b	40.75 ± 3.49 b	10.73 ± 0.91 b
WP8 + RA2	50.45 ± 2.80 d	18.55 ± 0.82 b	5.58 ± 0.55 c	3.48 ± 0.42 b	37.23 ± 1.57 b	8.83 ± 1.33 c

Emergence rate of tomato was assessed at 17 days after sowing (DAS), growth indicators including plant height, root length, stem diameter, aboveground and root dry weight were obtained by routine methods at 40 DAS. Values are means of three independent replicates ± SE. Different letters mean significant differences (Duncan's multiple range test, at $p < 0.05$) among treatments

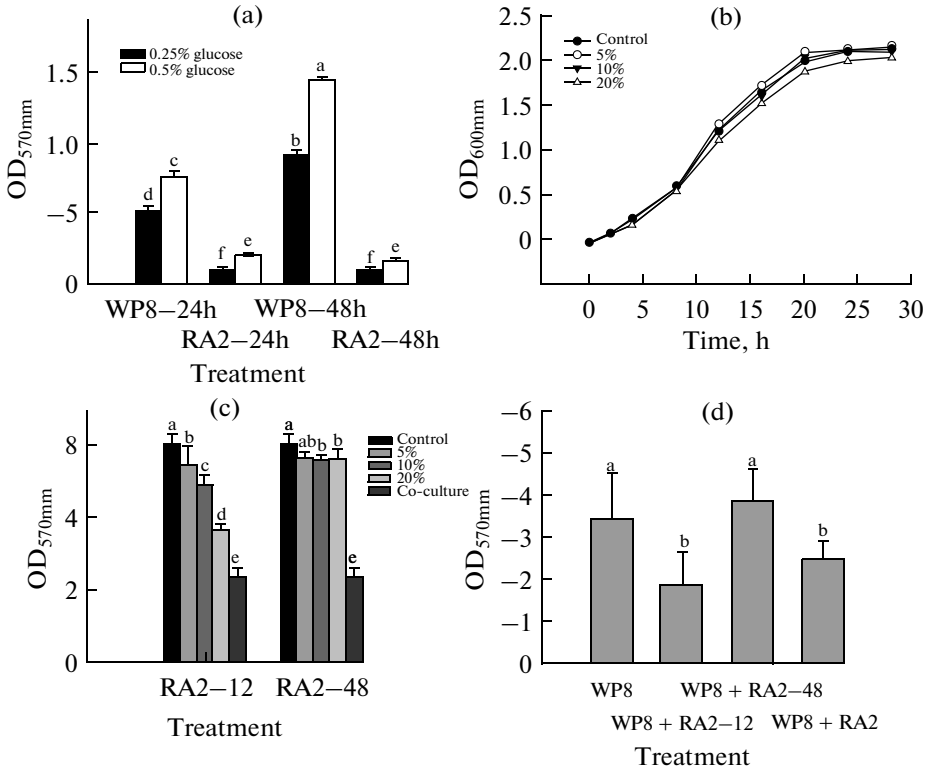

Fig. 2. (a) In vitro biofilm formation abilities of *B. pumilus* WP8 and *E. persicinus* RA2. WP8 (RA2)–24 h and –48 h indicate that WP8 (RA2) were incubated in 96–well polystyrene plates for 24 and 48 h, respectively. (b) Effects of RA2–12 at different concentrations on WP8 growth, (c) effects of RA2–12 and RA2–48 at different concentrations, as well as RA2 ceils on biofilm formation by WP8. RA2–12 and –48 represent filtered supernatants of RA2 after 12 and 48 h of culture, respectively. (d) In vivo effects of RA2–12 and RA2–48 on biofilm formation on tomato roots. Different letters mean significant differences (Duncan's multiple range test, at $p < 0.05$) among treatments.

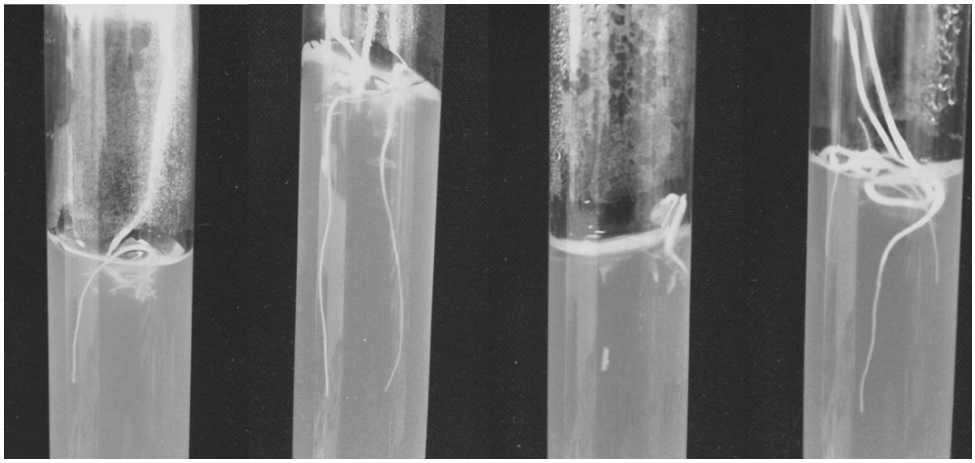

Fig. 3. Effects of different seed treatments on tomato seedling growth at 12 days after transplant. Treatments, from left to right, are control (no inoculation), WP8, WP8 + RA2–12 and WP8 + RA2–48.

ary metabolites of RA2, such as amino acids and organic acids. Interestingly, D-amino acids [22], *cis*-2-decenoic acid [23], *S*-phenyl-L-cysteine and its breakdown products, and diphenyl disulfide [24] were shown to be effective biofilm inhibitors. Moreover, supernatants of RA2–12 still inhibited biofilm formation in WP8 at high temperatures of up to 100°C (Fig. 5c), indicating that inhibitor(s) in RA2–12 were not proteinaceous. LC-MS data revealed two putative metabolites, D-glutamine in RA2–12 (Plot A in

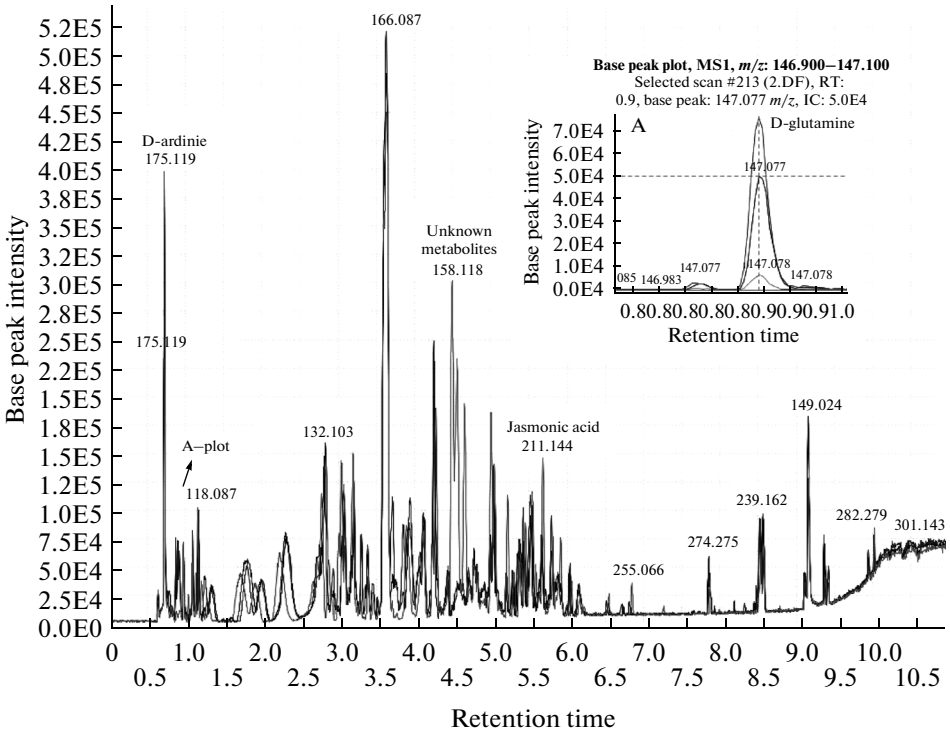

Fig. 4. LC–QTOF MS-based comparative metabolite profiling among RA2–12 (blue), RA2–48 (green) and control (red, TSB blank culture medium). Colors of curves in A–plot are different (red, blue and green represent RA2–12, control and RA2–48, respectively). Mass values corresponding to different peaks were searched in the METLIN database (http://metlin.scripps.edu/metabo_search.php) by selecting $[M + H]^+$ ions for automated identification with a mass tolerance of 0.05 Da.

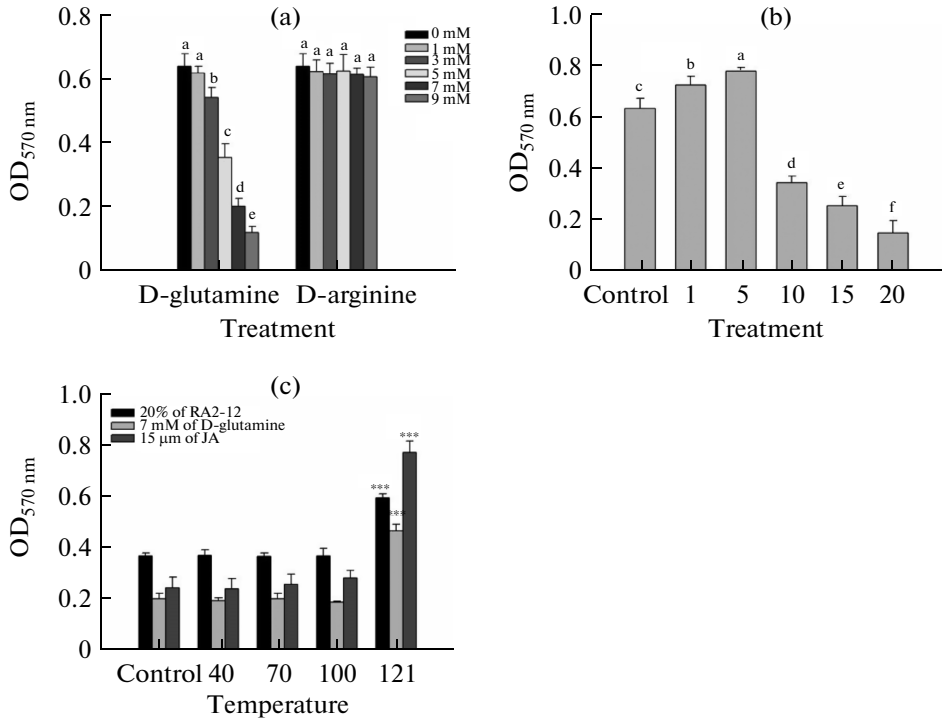

Fig. 5. (a) Effects of D-arginine and D-glutamine, as well as (b) Jasmonic acid on biofilm formation by WP8. Different letters mean significant differences (Duncan's multiple range test, at $p < 0.05$) among treatments. (c) Heat resistance of RA2–12, D-glutamine and JA for inhibition of biofilm formation by WP8. Three asterisks (* * *) indicate significant differences (Duncan's multiple range test, at $p < 0.01$) among treatments.

Fig. 4) and JA in RA2–48. The former is a D-amino acid and the latter, produced by *B. pumilis* [25, 26], is a well-known signal substance for plant defense [27, 28]. Both were confirmed by comparison with pure compounds (Fig. 5), and thus we deduce that biofilm inhibition may not contribute to D-arginine but D-glutamine production. Low concentration of JA promoted biofilm formation by WP8 (Fig. 5b), as shown in *Staphylococcus aureus* [29]. Although high concentrations of JA inhibited biofilm formation by WP8, JA amounts added in vitro did not exceed the threshold estimated based on bacterial yield of JA [25, 30]. This may explain why biofilm formation decreased dramatically in the mixture ofWP8 and RA2. In field conditions, biological control and/or growth promotion effects by PGPR mixtures may disappear while the organisms change from biofilm to planktonic states in root system. Moreover, the fermentation broth of RA2–48 was more viscous than that of RA2–12, due to abundant extracellular polysaccharides (data not shown), which may also contribute to the rescue of robust biofilm formation by WP8 [31, 32]. In addition, glucose promoted biofilm formation by WP8 (Fig. 2a) in agreement with previous reports [33, 34], further supporting the claim above.

In conclusion, we provide the first evidence that PGPR mixtures may not display synergistic; effects, by inhibition of biofilm formation rather than growth inhibition. These findings should be taken into account in the design of PGPR mixtures. Further studies should be carried out using appropriate RA2 mutants to confirm this phenomenon. In addition, the GFP-labeled WP8 strain should be used to assess biofilm formation *in situ* more accurately.

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