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ORIGINAL PAPER

Biodegradation of soil-applied pesticides by selected strains of plant growth-promoting rhizobacteria (PGPR) and their effects on bacterial growth

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Abstract A laboratory study was conducted to investigate the influence of four PGPR strains on the degradation of five soil applied pesticides and their effects on bacterial growth. Interactions of *Bacillus subtilis* GB03, *Bacillus subtilis* FZB24, *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* SE34 with two concentrations of acibenzolar-*S*-methyl, metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam in liquid culture and soil microcosm were studied. The degradation of acibenzolar-*S*-methyl by all PGPR tested in low and high concentration, was 5.4 and 5.7 times, respectively, faster than that in non-inoculated liquid culture medium. At the end of the 72-h liquid cultured experiments, 8–18, 9–11, 15–36 and 11–22% of metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam, respectively, had disappeared from PGPR inoculated medium. Under the soil microcosm experimental conditions, the half-lives of acibenzolar-*S*-methyl incubated in the presence of PGPR strains spiked at 1.0 and 10.0 mg kg^{-1} were 10.3–16.4 and 9.2–15.9 days, respectively, markedly lower compared with >34.2 days in the control. From the rest pesticides studied degradation of propamocarb hydrochloride and thiamethoxam was enhanced in the presence of *B. amyloliquefaciens* IN937a and *B. pumilus* SE34. Acibenzolar-*S*-methyl, propamocarb hydrochloride and thiamethoxam significantly increased the PGPR growth. However, the stimulatory effect was related to the level of pesticide spiked.

Keywords Pesticides · Biodegradation · Plant growth-promoting rhizobacteria (PGPR) · *Bacillus* sp. · LC–MS/MS

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are beneficial naturally occurring soil bacteria that colonize the rhizosphere and plant roots resulting in enhancement of plant growth and protection against certain plant pathogens (Van Loon 2007). These rhizobacteria exert their beneficial effects through either direct or indirect mechanisms. Direct plant growth-promoting mechanisms induced by PGPR include the production of plant hormones like auxins, gibberellins and cytokinins, nitrogen fixation,

phosphate solubilization, and uptake of essential plant nutrients (Spaepen et al. 2009; Vessey 2003). PGPR can also indirectly induce plant growth by protecting plants against pathogens, a process known as biocontrol activity. Different indirect mechanisms such as induced systemic resistance, production of antimicrobial compounds, and competition for nutrients and colonization sites with pathogens have been described (Kloepper et al. 2004).

Bacteria identified as PGPR have diverse taxonomy and include strains of the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Gordonia*, *Klebsiella*, *Paenibacillus*, *Pseudomonas*, *Serratia*, among others (Glick 1995; Hong et al. 2011). *Bacillus* and *Pseudomonas* spp. have a wide distribution and are the most extensively studied. In recent years, several PGPR-based products became commercially available in many countries, and more are currently under development (Choudhary and Johri 2009). Most of these products contain species of the genus *Bacillus* which form endospores that confer population stability during formulation and storage. Among the bacilli, strains of *Bacillus subtilis* are the most widely used PGPR because they are able to produce a broad spectrum of antibiotics, and effectively colonize the rhizosphere and control soil-borne pathogens (Cazorla et al. 2007). Currently, there is an increasing interest in testing PGPR-based products in agricultural crop production systems. These products are mainly applied as seed treatment, soil amendment or soil drench at the time of seeding or immediately after transplanting, to promote plant growth and effectively suppress several diseases in a number of crops (Kloepper et al. 2004).

In addition to biopesticides, many soil-applied synthetic pesticides are used against weeds, pests and pathogens during conventional or intergraded crop protection programs. Under actual agricultural practices, different groups of pesticides, such as, insecticides, fungicides, herbicides, and commercially available PGPR are often simultaneously or consecutively applied interacting with each other. Furthermore, much attention has recently been paid on bioremediation of contaminated soils with PGPR (Huang et al. 2004; Jiang et al. 2008). *Pseudomonas, Azospirillum, Agrobacterium, Bacillus, Enterobacter,* and *Flavobacterium* are some of the genera which include PGPR strains able to degrade organic and inorganic contaminants in soil (Zhuang et al. 2007).

To the best of our knowledge, limited data are available on in vitro biodegradation of soil-applied pesticides by PGPR strains and their effects on bacterial growth (Osman et al. 2008). On the other hand, several works have focused on the effect of pesticides on the indigenous soil microbial community (Saeki and Toyota 2004; Bending et al. 2007; Wang et al. 2008) but little is known regarding the effect of soil-applied pesticides on the introduced PGPR populations. There is, therefore, an increasing concern on the interactions from the combined application of PGPR and synthetic pesticides.

In the present study our objective was to investigate the interactions of soil-applied pesticides with certain bacilli PGPRs both of which are applied to protect and promote tomato production. Pesticides and PGPRs interactions were tested in liquid medium and in soil microcosm. Two PGPR formulated strains of *Bacillus subtilis*, GB03 (Companion) and FZB24 (FZB24 li.), two laboratory PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* SE34, and five pesticides metribuzin, napropamide (herbicides), propamocarb hydrochloride, acibenzolar-*S*-methyl (fungicides) and thiamethoxam (insecticide), which among others are used in tomato crop protection, were studied.

Materials and methods

Chemicals and reagents

Analytical grade acibenzolar-*S*-methyl (99.5%) was obtained from Wako Pure Chemical (Osaka, Japan), metribuzin (99.8%), napropamide (99.9%) and thiamethoxam (99.7%) were purchased from Riedel-de Haën (Seelze, Germany) and propamocarb hydrochloride (98%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ethyl acetate, acetonitrile, methanol and water, all HPLC grade, were obtained from Merck (Darmstadt, Germany), formic acid (99%) and sodium chloride (99.8%) were purchased from Carlo Erba (Milano, Italy) and Riedel-de Haën (Seelze, Germany), respectively. Stock standard solutions containing 1 mg ml^{-1} of each compound were prepared by dissolving accurately weighed amounts in methanol and stored in darkness at −25°C. Working standard solutions were made in methanol for the preparation of fortified broth

samples and also in mobile phase for the construction of calibration curves. In soil microcosm biodegradation study commercial formulations of the pesticides were used (Table 1).

Soil samples and media

The soil used in the present study was collected from the surface layer (0–15 cm depth) of a field with no pesticide application history located in the central Greece (04307473°N, 00354535°E). Soil samples were dried at room temperature, mixed, and then passed through a 2-mm sieve and stored at 4°C prior to use. The physical and chemical properties of the soil were: pH 7.7, organic matter 0.8%, maximum water holding capacity 57.3%, sand 35.6%, silt 24.8% and clay 39.6%.

The compositions of the media used were as follows—tryptic soy broth medium (TSB, Difco Laboratories, Detroit, MI, U.S.A.): pancreatic digest of casein 17.0 g, papaic digest of soybean 3.0 g, dextrose 2.5 g, sodium chloride 5.0 g, dipotassium phosphate 2.5 g, distilled water 1 l, pH 7.3 and tryptic soy agar medium (TSA, Difco Laboratories, Detroit, MI, U.S.A.) used for agar plates: pancreatic digest of casein 15.0 g, papaic digest of soybean 5.0 g, sodium chloride 5.0 g, agar 15.0 g, distilled water 1 l, pH 7.3.

Sources of PGPR strains and inoculum preparation

The PGPR used in these studies were *Bacillus amyloliquefaciens* strain IN937a, *Bacillus pumilus* strain SE34, *Bacillus subtilis* strain FZB24, and *Bacillus subtilis* strain GB03. The PGPR strains, *B. amyloliquefaciens* IN937a and *B. pumilus* SE34, were obtained from the culture collection of the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL). The commercially available PGPR strains of *B. subtilis*, GB03 (Companion) and FZB24 (FZB24 li.) were provided by Growth Products (New York, USA) and ABiTEP GmbH (Berlin, Germany), respectively. The bacteria were maintained in TSB medium amended with 20% glycerol at −80°C for long-term storage. Bacterial cell suspensions were prepared by streaking each PGPR strain taken from ultracold storage onto TSA plates, incubating the plates at 28°C for 48 h to check for purity, and then single colonies were transferred to fresh TSB flasks and incubated under shaking at 28°C for 24 h. The bacterial cultures were centrifuged at 8,000 rpm for 10 min and the precipitate was resuspended in sterile distilled water to obtain a final density of about 1×10^9 CFU (colony forming units) mL^{-1}. Bacterial concentration was determined as described below by the plate counting method, in terms of CFU.

Biodegradation of pesticides in liquid medium

For biodegradation studies, the TSB medium was fortified with each of the pesticides at following levels: acibenzolar-*S*-methyl (1.0 and 10.0 mg l^{-1}), metribuzin (0.25 and 2.50 mg l^{-1}), napropamide (2.0 and 20.0 mg l^{-1}), propamocarb hydrochloride (60.0 and 600.0 mg l^{-1}) and thiamethoxam (0.2 and 2.0 mg l^{-1}). The fortification levels represent one and ten times concentrations of the recommended field doses of the compounds when applied to 10 cm depth of soil. Each PGPR suspension (1 ml) was inoculated into the sterilized Erlenmeyer flasks containing 100 ml of TSB medium and spiked with 0.10 ml of respective pesticide stock solution dissolved in methanol providing the above final concentrations (the final methanol concentration in media was 0.10%). Uninoculated spiked flasks were set as controls. All flasks were incubated in the dark at 30°C and 150 rpm on a rotary shaker. At intervals of 0, 10, 24, 30, 48 and 72 h, samples were removed

Table 1 List of the commercial formulations of the pesticides used in soil biodegradation study

Pesticide	Formulation	Group	Company
Acibenzolar-*S*-methyl	Bion 50 WG	Fungicide	Syngenta Crop Protection Inc.
Metribuzin	Sencor 70 WG	Herbicide	Bayer Hellas S.A.
Napropamide	Devrinol 45 SC	Herbicide	Alfa Agricultural Supplies S.A.
Propamocarb hydrochloride	Previcur N 72.2 SL	Fungicide	Bayer Hellas S.A.
Thiamethoxam	Actara 25 WG	Insecticide	Syngenta Hellas S.A.

and the bacterial growth and pesticide degradation rates were determined.

Biodegradation of pesticides in soil microcosm

Soil microcosm experiment was contacted with sterilized soil in order to exclude the interaction with the endogenous microbial community. For the preparation and treatment of sterile soil samples, all materials were sterilized before utilization and all preparations were done under a laminar flow hood. The soil was sterilized by autoclaving on three consecutive days at 121°C for 60 min each and sterility of soil was determined by the dilution plate technique. Subsamples (200 g) of sterile soil were weighed, placed in 500 ml Erlenmeyer flasks, and then treated with the appropriate pesticide concentrations, under sterile conditions. After mixing, the bacterial suspension of each strain was inoculated into soil to give a final concentration of about 2×10^7 CFU g^{-1}. The inoculum was thoroughly mixed into the soil under sterile conditions, and the moisture content was adjusted by the addition of sterile distilled water to 50% of its maximum water holding capacity. Samples of sterilized soil inoculated with bacteria and uninoculated spiked flasks were kept as controls. All soil samples were incubated at 30°C in the dark. Sterilized distilled water was added at regular intervals throughout the incubation period to compensate for loss of water by evaporation. Soil samples were periodically removed aseptically and bacterial population and pesticide residues were measured.

Extraction procedure and LC–MS/MS analysis

Liquid medium. 5 ml of liquid medium were supplemented with 1 g NaCl and extracted twice with 10 and 5 ml ethyl acetate, respectively. After centrifugation at 4,000 rpm for 5 min, 10 ml of supernatant were concentrated to dryness by use of nitrogen stream. The residues were reconstituted in 1 ml mobile phase and transferred to autosampler vials for the instrumental analysis in LC–MS/MS system.

Soil. 5 g (dry weight) of soil was twice extracted with 10 and 5 ml acetonitrile, respectively, followed by centrifugation for 5 min at 8,000 rpm. 10 ml of the supernatant were concentrated to dryness by use of nitrogen stream at 32°C, and redissolved in 1 ml mobile phase. The residues filtered through 0.45 μm PTFE filter and were taken for instrumental analysis. The above extraction methods were used for all the pesticides apart from propamocarb hydrochloride. The extraction of this compound was carried out according to a modified version of the QuEChERS method, as described by Anastassiades et al. (2007).

Liquid medium and soil extracts were analyzed by using a LC–MS/MS system consisting of a Surveyor Autosampler (Thermo Finnigan, USA), a Surveyor LC Pump connected with a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved using a Hypurity-C18 (Thermo Finnigan, USA) analytical column (4.6 mm × 150 mm, 5 μm) thermostated at 30°C. 10 μl portion was injected and the elution was conducted under isocratic conditions with 0.4 ml min^{-1} acetonitrile:water (50:50, v/v) with 0.15% (v/v) formic acid. The ESI source was set at the positive ionization mode. The operating conditions for ESI were as follows: spray voltage 4,000 V; capillary temperature 380°C; nitrogen as the sheath gas (35 kPa); auxiliary gas pressure (20 kPa). The quantification was done using selected reaction monitoring mode. The precursor and fragmentation ions selected for quantification are presented in Table 2.

Enumeration of bacterial counts

The bacterial populations were determined using serial dilutions, plating, and counting the CFUs developed on the TSA plates. An aliquot (1 ml) of the broth culture was removed aseptically after 0, 10, 24, 30, 48 and 72 h of incubation, added to 9 ml of sterile phosphate buffer (10 mM, pH 7.0) and mixed vigorously by vortexing. The resulting suspension was serially diluted tenfold in sterile phosphate buffer and 100 μl of the serial dilutions 10^{-4}–10^{-6} were plated on TSA plates. The colonies that appeared in 48 h of incubation at 28°C on the plates were counted as CFU ml^{-1}.

Introduced bacterial populations into the sterile soil samples were measured as CFU g^{-1}, at intervals of 0, 7, 14, 21, 28 and 35 days, by transferring 1 g (dry weight) of soil from each flask into a sterile flask, containing 9 ml of a sterile solution of sodium hexametaphosphate (1.66 g l^{-1}, pH 7) (Jézéquel

Table 2 Retention time of the studied compounds, precursor ions and product ions used for quantification and confirmation during LC–MS/MS analysis

Pesticide	R.T. (min)	Precursor ion (*m/z*)	Quantification ion (*m/z*)	Confirmation ion (*m/z*)
Acibenzolar-*S*-methyl	15.89	211	136	140
Metribuzin	7.93	215	187	84
Napropamide	18.21	272	129	171
Propamocarb hydrochloride	3.37	189	102	144
Thiamethoxam	5.01	292	211	181

R.T. retention time

et al. 2005). Soil samples were shaken at 150 rpm for 1 h and a suspension of 1 ml was taken from each flask for serial tenfold dilutions and plating in TSA plates. The samples were plated in triplicates and incubated at 28°C for 48 h (Yan et al. 2003).

Method validation

The repeatability and accuracy of the analytical methods were evaluated with the analysis of liquid culture samples fortified at 0.005, 0.05, 0.5, 1.0, 10.0 mg l^{-1} and soil samples fortified at 0.005, 0.05, 0.1, 2.0, and 10.0 mg kg^{-1}. All recovery tests were done in triplicate. Calibration curves were prepared using external working standard solutions. The limit of detection (LOD) of each solute were determined as the lowest concentration giving a signal-to-noise ratio of 3. The limit of quantification (LOQ) in both cases (medium and soil) was determined as the lowest concentration of a given compound giving a response that could quantified with a relative standard deviation (RSD) lower than 20% (Vryzas and Papadopoulou-Mourkidou 2002).

Data analysis

FOCUS work group on degradation suggests that the visual assessment combined with the Chi^2 test is to be judged for the fit of acceptability of each model (FOCUS 2006). On this basis and since pesticide degradation data were fitted to first-order kinetics. The half-life ($t_{1/2}$) values were estimated based on first-order dissipation kinetics, $C_t = C_0 e^{-kt}$, where C_t is the concentration in the soil after time t, C_0 is the apparent initial concentration, and k and t are the rate constant (d^{-1}) and degradation period in days, respectively. The half-life values were calculated from the linear equation obtained from the regression analysis of the chemical data and time using JMP (JMP, SAS Institute, Cary, NC). First-order kinetics failed to describe the degradation of metribuzin which showed biphasic degradation patterns and the bi-exponential model (1) was used instead.

$$C_t = C_1 \times e^{-k_1 \times t} + C_2 \times e^{-k_2 \times t} \quad (1)$$

where C_t is the recovered amount of metribuzin at t days, C_1 and C_2 are the amounts of metribuzin at $t = 0$ and k_1 and k_2 are the disappearance rate constants for the first and second compartment, respectively. C_1 and C_2 is approximately equal to C_0 (total amount of metribuzin applied at $t = 0$) (FOCUS 2006).

Data were submitted to one-way analysis of variance (ANOVA) $P < 0.05$, and Duncan's multiple range test was used for means comparison. The statistical analysis was supported by SPSS 17.0 (SPSS, Chicago, USA).

Results and discussion

Validity of analytical procedure

Mean recovery values obtained for acibenzolar-*S*-methyl, metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam were 85, 97, 95, 86, 114% and 80, 91, 90, 81, 89% for TSB and soil, respectively. The relative standard deviation values (RSDs%) of all % mean recovery values were <11% at the 0.05–10.0 mg kg^{-1} fortification levels and <15% at level of 0.005 mg kg^{-1}. The accuracy (as recoveries) and precision (as repeatability) of the method were acceptable since recovery values were >80% and respective RSDs were <15% for all solutes and at all fortification levels (SANCO 2009). LC–MS/MS chromatograms from the analysis of fortified (0.1 mg kg^{-1}) soil samples are shown in Fig. 1. The LOQ and LOD levels for all solutes for

both substratum (soil and medium) were set at 0.005 mg kg^{-1} (or mg l^{-1}) and 0.001–0.002 mg kg^{-1} (metribuzin and napropamide at 0.001, acibenzolar-*S*-methyl, propamocarb hydrochloride and thiamethoxam at 0.002 mg kg^{-1}), respectively.

Degradation of pesticides by PGPR strains in liquid culture medium

The degradation kinetics of acibenzolar-*S*-methyl in TSB medium by four PGPR strains is presented in Fig. 2a1, a2. After 24 h of incubation, 58–100% of acibenzolar-*S*-methyl was degraded by all PGPR strains tested, compared to 5–7% in the uninoculated control. *B. pumilus* SE34 showed the highest degradation rate among these strains, with 100% degradation of acibenzolar-*S*-methyl within 24 h at both concentrations tested. *B. amyloliquefaciens* IN937a and *B. subtilis* FZB24 degraded acibenzolar-*S*-methyl with a similar efficiency 78–79 and 71–74%, respectively. The degradation rates of acibenzolar-*S*-methyl by *B. subtilis* GB03 were 58 and 60% at 1.0 and 10.0 mg l^{-1}, respectively. After incubation for 48 h, more than 98% of the acibenzolar-*S*-methyl initially added to the medium was removed by the bacterial strains. During the same period, in the control flasks, the abiotic dissipation rates of acibenzolar-*S*-methyl were 17 and 16% at 1.0 and 10.0 mg l^{-1}, respectively. The degradation was obviously accelerated by the addition of these four strains. The initial acibenzolar-*S*-methyl concentrations were completely degraded by all PGPR strains at the end of the incubation period. To our knowledge, this is the first report of PGPR belonging to genus *Bacillus* with the ability to degrade rapidly acibenzolar-*S*-methyl in liquid medium. Dissipation due to decrease of pH values in the presence of PGPR strains was excluded since pH values were slightly reduced (from 7.4 to 6.6) in all cases. Moreover, all pesticides are more stable at pH 5–7 according to the pesticide properties database of University of Hertfordshire (FOOTPRINT: creating

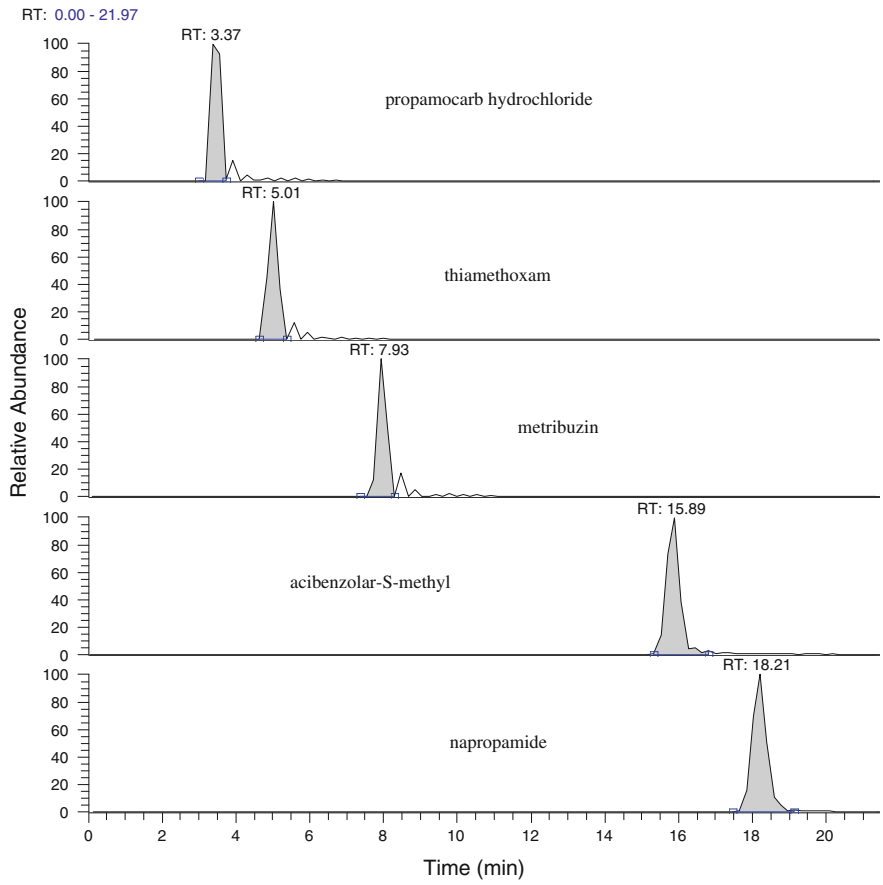

Fig. 1 LC-MS/MS chromatograms of the studied pesticides obtained from fortified (0.1 mg kg^{-1}) soil samples

tools for pesticide risk assessment and management in Europe 2011).

The degradation patterns of metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam in TSB cultures inoculated with the PGPR strains are shown in Fig. 2b1, b2, c1, c2, d1, d2, e1, e2. No appreciable dissipation of the tested pesticides was observed in the non-inoculated control samples. Concerning the pesticides tested, after 72 h of incubation metribuzin was degraded 14–18 and 8–12% by *B. subtilis* FZB24 and *B. subtilis* GB03, respectively and napropamide was degraded 9–11% by *B. subtilis* FZB24 (Fig. 2b1, b2, c1, c2). The degradation rates of metribuzin and napropamide by the other bacterial strains tested were not statistically different with the dissipation rates of the control samples. In a previous biodegradation study, from the 53 fungal species isolated from pesticide-contaminated and uncontaminated soil, only three species were able to remove more than 50% of metribuzin after 5 days of incubation in liquid medium (Bordjiba et al. 2001). The majority of the species were able to degrade partly the compound (between 15 and 40%) and some exhibited very low biodegradation potentialities (below 20%). Schilling et al. (1985) also reported degradation of metribuzin by pure cultures of five fungi. Grigg et al. (1997) reported that metribuzin was not degraded in liquid medium by mixed microbial culture isolated from soil. Study on microbial degradation of napropamide is very limited. Until now, no pure bacterial cultures that could degrade napropamide have been described in literature. In our study, 15% of the initially added propamocarb hydrochloride (60 mg l^{-1}) was removed by strain *B. amyloliquefaciens* IN937a and 22% by *B. pumilus* SE34 (Fig. 2d1). At the high spiking level these strains were capable to degrade 19 and 36% of propamocarb hydrochloride, respectively (Fig. 2d2). Our results are in agreement with previous findings reported by Knowles and Benezet (1981) who found little degradation of the fungicide propamocarb by all the soil microorganisms studied. The bacterial strains *B. amyloliquefaciens* IN937a, *B. pumilus* SE34 and *B. subtilis* FZB24 degraded 16–18, 19–22, and 11–14% of thiamethoxam, respectively, after 72 h of incubation (Fig. 2e1, e2). Recently, Pandey et al. (2009) have reported the isolation of *Pseudomonas* sp. strains 1G, 1W, and GP2 which were capable to degrade about 70% of thiamethoxam after 14 days of incubation.

To date, limited data are available on in vitro biodegradation of soil-applied pesticides by pure cultures of plant growth-promoting rhizobacteria, which constitute one of the main techniques involved in the integrated pest management. Previous studies by Osman et al. (2008) have suggested that 26–33% of dicofol was degraded after 3 days of incubation with the tested PGPR. The percentages of residual amount of bromoxynil from media enriched with seven isolates of PGPR ranged from 30 to 72% after incubation for 72 h (Askar et al. 2007).

Bacterial growth in medium supplemented with the pesticides

The growth of bacterial strains monitored by viable colony counts of bacteria in TSB medium after 0, 10, 24, 30, 48 and 72 h of incubation in the presence or absence of acibenzolar-*S*-methyl is shown in Fig. 3. Immediately after inoculation the initial number of bacterial colonies was 0.80–2.12 $\times$ 10^7 CFU ml^{-1}. Thereafter, the PGPR strains showed an exponential phase of growth and the number of colonies reached a maximum (1.06–2.20 $\times$ 10^9 CFU ml^{-1}) within 24–30 h of incubation. After this sharp increase in colony count the bacterial growth was gradually decreased to 0.94–1.74 and 0.74–1.36 $\times$ 10^9 CFU ml^{-1} after 48 and 72 h of incubation, respectively. This was in general agreement with Kim et al. (2004), report who found that the maximum growth of *Sphingomonas* sp. strain SB5 occurred in 24 h of incubation and then showed a declined trend in time-course degradation of carbofuran by SB5. The growth pattern of *B. pumilus* SE34 in the presence of 1.0 mg l^{-1} acibenzolar-*S*-methyl was almost the same as the growth shown in the unspiked control. When this compound tested at the spiking level of 10.0 mg l^{-1} the strain growth was stimulated by the presence of pesticide as the number of colonies was consistently higher from the control. The growth of *B. pumilus* SE34 was accompanied by concurrent degradation of acibenzolar-*S*-methyl, suggesting that the bacterial strain was growing at the expense of the compound. Also the number of colonies in treatments with *B. amyloliquefaciens* IN937a and *B. subtilis* FZB24 during the 72 h of incubation was increased slightly compared to the control. In case of

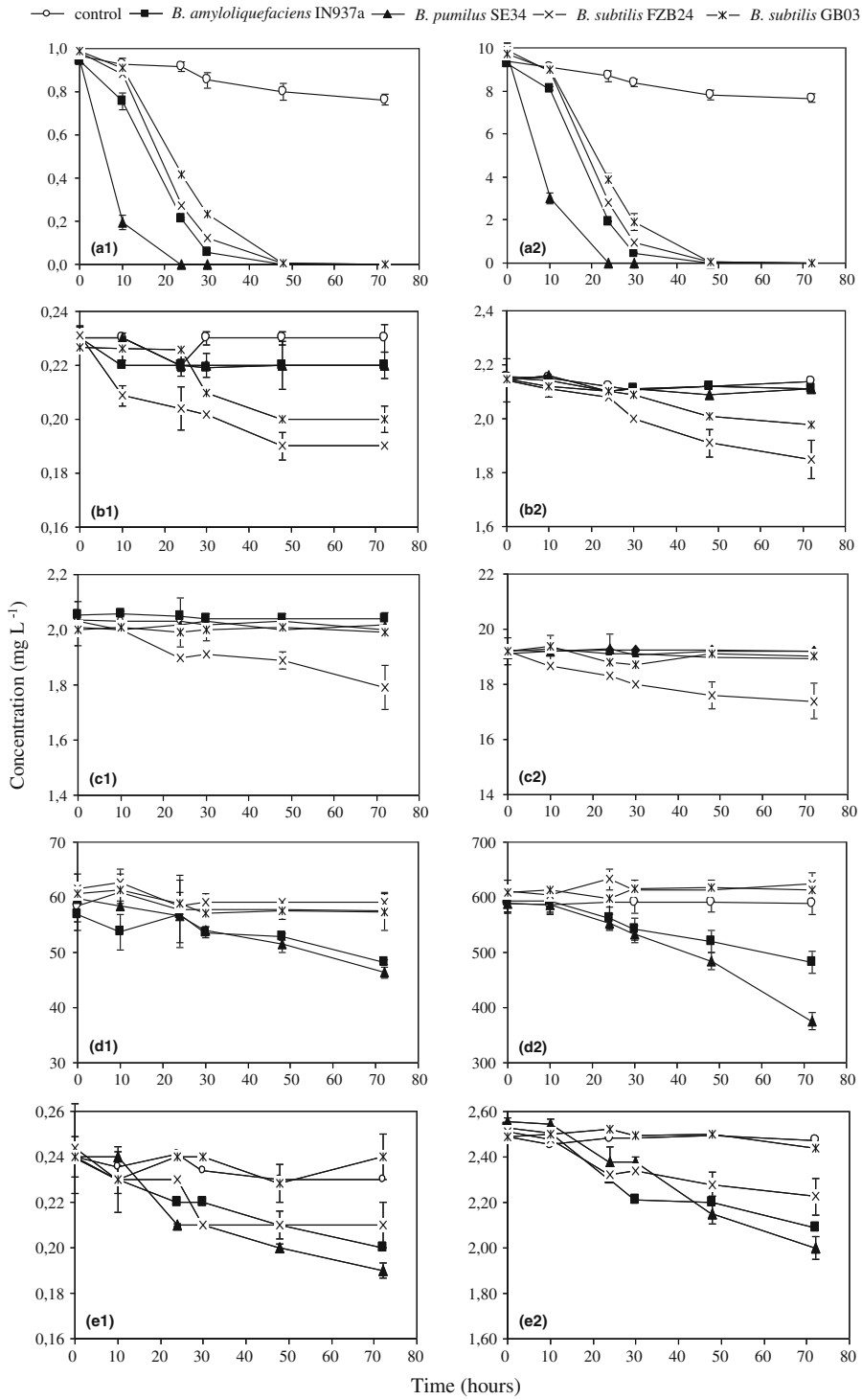

Fig. 2 Degradation of acibenzolar-*S*-methyl (**a1**, **a2**), metribuzin (**b1**, **b2**), napropamide (**c1**, **c2**), propamocarb hydrochloride (**d1**, **d2**), and thiamethoxam (**e1**, **e2**) at one and ten times the recommended field doses of the compounds by PGPR strains in tryptic soy broth (TSB) medium. Each value is the mean of three replicates with *error* bars representing the standard deviation of the mean

B. subtilis GB03 the addition of acibenzolar-*S*-methyl did not exhibit any significant effect on the growth of bacteria.

Concerning the other pesticides tested, the growth kinetics of bacterial strains presented an initial exponential growth. The number of colonies

increased until a maximum and then a slight decrease was observed till the 72nd h in all cases irrespective to pesticide use. In particular, the data revealed that all bacterial strains in liquid TSB medium amended with one and ten times the recommended dose of metribuzin showed a fine growth pattern compared to the untreated control, with the exception of *B. pumilus* SE34 at the higher fortification level of 2.5 mg l^{-1}, which led to lower growth values until the end of the experiment. The addition of 0.2 and 2.0 mg l^{-1} thiamethoxam decreased the bacterial growth of *B. subtilis* GB03 and caused a slight increase in the growth of rest strains. Furthermore, the effect of the herbicide napropamide was negligible and the bacteria grew well in both concentrations tested. The stimulatory effect of the carbamate fungicide propamocarb hydrochloride at the fortification levels of 60 and 600 mg l^{-1} on populations of *B. ayloliquefaciens* IN937a and *B. pumilus* SE34 was also observed. Inhibitory effect of the herbicide metribuzin and the insecticide thiamethoxam at higher the recommended doses on the plant growth-promoting rhizobacterium *Rhizobium* sp. strain MRL3 grown in liquid medium has recently been reported by Ahemad and Khan (2011).

Biodegradation studies in soil

In cases where data from soil degradation study were fitted to first-order kinetics, the coefficients of determination (r^2) for first-order fitting of pesticides in both concentrations were higher than 0.914. The bi-exponential model was used in order to describe the degradation process of metribuzin with coefficients of determination (r^2) higher than 0.981.

Compared to the total mass of biodegradable nutrients available to the soil the pesticides incorporated supply low concentration of nutrients and could be used as secondary substrate by PGPR strains. This implies that the pesticides tested are most likely degraded by "co-metabolism" (FOCUS 2006).

The half-life ($t_{1/2}$) values for the tested pesticides in various soil treatments are shown in Table 3. In the case of sterilized soils inoculated with the different PGPR strains, degradation kinetics of acibenzolar-*S*-methyl was very similar in soil treatments with the strains *B. amyloliquefaciens* IN937a, *B. subtilis* FZB24 and *B. subtilis* GB03. Degradation process in the presence of the three above mentioned PGPR strains was characterized by an average rate constant (k) of 0.046, 0.045 and 0.043 $days^{-1}$, respectively.

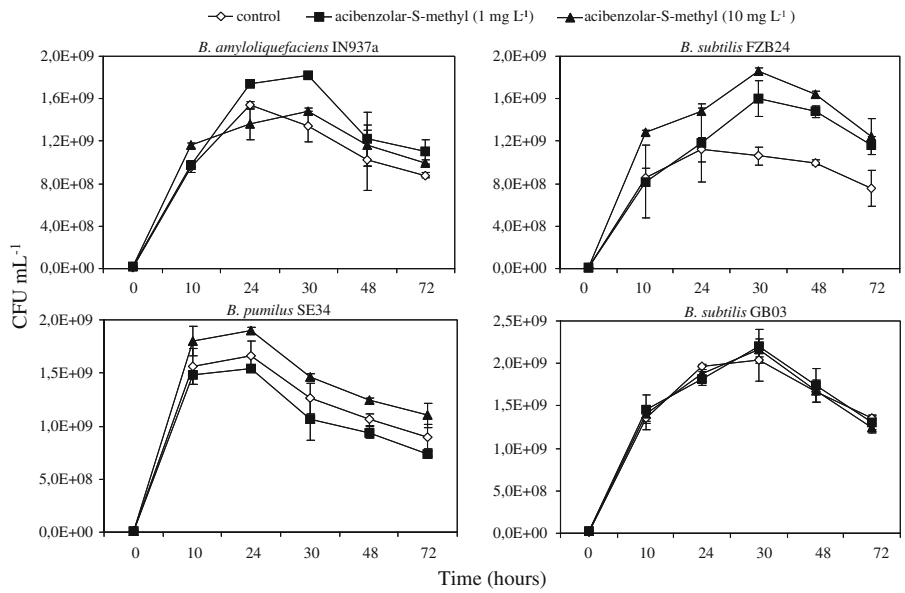

Fig. 3 Growth of four *Bacillus* sp. PGPR strains in tryptic soy broth (TSB) medium spiked with acibenzolar-*S*-methyl at levels of 1.0, 10.0 mg l^{-1}, and unspiked control. *Error bars* represent the standard deviation of the mean

Table 3 Half-life values ($t_{1/2}$) obtained from the soil degradation patterns of the compounds treated with the PGPR strains

$t_{1/2}$ (day)										
Soil treatment	Acibenzolar-*S*-methyl		Metribuzin		Napropamide		Propamocarb hydrochloride		Thiamethoxam	
	1 mg kg^{-1}	10 mg kg^{-1}	0.25 mg kg^{-1}	2.5 mg kg^{-1}	2 mg kg^{-1}	20 mg kg^{-1}	60 mg kg^{-1}	600 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}
Control[x]	38.8a[y]	34.2a	133.5a	141.3a	182.2a	228.9a	117.8a	123.1a	236.3a	268.9a
IN937a	15.1b	14.5b	129.8a	139.0a	180.3a	226.5a	91.8c	94.9c	219.1b	251.0b
SE34	10.3c	9.2c	128.1a	140.6a	176.7a	225.2a	104.9b	110.8b	212.9b	255.1b
FZB24	15.9b	14.7b	131.9a	138.4a	179.1a	225.7a	118.2a	121.9a	216.1b	253.5b
GB03	16.4b	15.9b	132.7a	138.0a	178.3a	222.4a	115.0a	123.9a	233.6a	265.5a

[x] Control: non-inoculated sterilized soil; IN937a: sterilized soil inoculated with *B. amyloliquefaciens* IN937a; SE34: sterilized soil inoculated with *B. pumilus* SE34; FZB24: sterilized soil inoculated with *B. subtilis* FZB24; GB03: sterilized soil inoculated with *B. subtilis* GB03

[y] Mean values followed by different letters in a column indicate significant differences among treatments according to Duncan's multiple range test ($P = 0.05$)

The results also showed that the addition of PGPR strain *B. pumilus* SE34 more efficiently degraded acibenzolar-*S*-methyl in sterilized soil as compared to soils inoculated with the rest strains and control. The average rate constant of acibenzolar-*S*-methyl disappearance by *B. pumilus* SE34 was 0.071 days^{-1}. The time within which the initial concentration of acibenzolar-*S*-methyl was reduced by 50% was 15.1, 10.3, 15.9, 16.4 days and 14.5, 9.2, 14.7, 15.9 days for *B. amyloliquefaciens* IN937a, *B. pumilus* SE34, *B. subtilis* FZB24 and *B. subtilis* GB03 at spiking levels of 1.0 and 10.0 mg kg^{-1}, respectively (Table 3). In the non-inoculated controls, the $t_{1/2}$ values were 38.8 and 34.2 days at low and high concentration, respectively, markedly higher compared to all other treatments (Table 3). This is in line with our previous studies which showed a rapid degradation of the compound by PGPR strains in TSB medium. As in the TSB medium case *B. pumilus* SE34 caused the highest degradation rates of acibenzolar-*S*-methyl among the PGPR strains tested.

Concerning the other pesticides tested metribuzin and napropamide dissipated at slow rates with the respective $t_{1/2}$ ranging from 128.1 to 141.3 days and 176.7 to 228.9 days, respectively (Table 3). Metribuzin exhibited a biphasic dissipation curves consisted of a phase of a more rapid dissipation occurring during the first 14 days of incubation followed by a second phase of slower dissipation rate. Our results are in accordance with previous investigations which reported that the degradation of metribuzin in soil samples was described using two-compartment model with a very rapid degradation rate at the beginning followed by a slow prolonged dissipation (Henriksen et al. 2004). The half-lives of metribuzin at low fortification level were 129.8, 128.1, 131.9 and 132.7 days in soil amended with *B. amyloliquefaciens* IN937a, *B. pumilus* SE34, *B. subtilis* FZB24 and *B. subtilis* GB03 and at high level they were 139.0, 140.6, 138.4 and 138.0 days, respectively (Table 3). The results also showed that the $t_{1/2}$ of metribuzin in un-inoculated soil were 133.5 and 141.3 days at low and high concentration, respectively. In the case of herbicide napropamide $t_{1/2}$ values ranging from 176.7 to 180.3 days and from 222.4 to 226.5 days at levels of 2.0 and 20.0 mg kg^{-1}, respectively, in various PGPR treatments compared to 182.2 and 228.9 days in non-inoculated soil (Table 3). In both, metribuzin and napropimide cases insignificant differences

($P > 0.05$) in half-lives of control and PGPR treated samples were observed suggesting no degradation effect of PGPR strains in the overall dissipation of those pesticides in soil samples (Table 3). The calculated $t_{1/2}$ values of metribuzin were within the range (107–144 days) reported in a previous degradation study with sterile soil samples (Khoury et al. 2006). In addition, $t_{1/2}$ values for napropamide were in general agreement with Guo et al. (2008), who reported that the degradation half-life of napropamide in sterilized soil was almost threefold of that in non-sterilized soil. Napropamide degradation in natural soil is slow, with a reported half-life of 56–84 days in laboratory incubations (Wauchope et al. 1992).

Degradation of propamocarb hydrochloride by *B. amyloliquefaciens* IN937a was more rapid followed by *B. pumilus* SE34, with the respective $t_{1/2}$ values ranging from 91.8 to 94.9 days and from 104.9 to 110.8 days, respectively. Insignificant differences in the dissipation rates of propamocarb hydrochloride among the control and the samples treated with *B. subtilis* FZB24 and *B. subtilis* GB03 were observed. *B. amyloliquefaciens* IN937a, *B. pumilus* SE34 and *B. subtilis* FZB24 were able to degrade thiamethoxam with $t_{1/2}$ values ranged from 219.1 to 251.0 days, 212.9 to 255.1 days and 216.1 to 253.5 days at 0.2 and 2 mg kg^{-1}, respectively. Insignificant differences in the dissipation rates of thiamethoxam were observed between control and soil treated with *B. subtilis* GB03 with respective $t_{1/2}$ values ranged from 236.3 to 268.9 days and 233.6 to 265.5 days in both concentrations tested (Table 3).

Our results showed that the degradation of herbicides metribuzin and napropamide by *Bacillus* sp. PGPR strains in liquid and soil environments were different. Specifically, the inoculation of sterilized soil samples had no significant effect on metribuzin and napropamide dissipation. Previous studies have also reported the different degradation potential of bacterial strains in sterile soil and broth culture (Goswami and Singh 2009). The inoculum density is an important factor determining the efficient biodegradation of applied pesticides (Karpouzas et al. 2005; Ramadan et al. 1990). Apart from biological factors many abiotic parameters have been shown to effect on the bacterial degradation of pesticides in soil. Properties of soil like organic matter content, pH, temperature, soil texture, and pesticide concentration have influence on the biodegradation process (Liu et al. 1990; Singh et al. 2006).

Effect of pesticide applications on PGPR populations during soil studies

Soil samples (1 g each) were taken at intervals of 0, 7, 14, 21, 28, 35 days and number of colonies were measured. Each bacterial strain was mixed into the soil corresponding to inoculum densities of about 2.0×10^7 CFU g^{-1} dry soil on day zero. Populations of *B. amyloliquefaciens* IN937a, *B. subtilis* FZB24, and *B. subtilis* GB03 in control soil samples showed a slight increase up to 14 days incubation and then gradually decreased reaching to 1.80×10^7, 1.63×10^7, and 1.50×10^7 CFU g^{-1}, respectively, after 35 days of incubation, while the number of *B. pumilus* SE34 colonies was maintained at the same level as the initial inoculation density (Fig. 4). Similarly, Yan et al. (2003) reported that population densities of *B. pumilus* SE34, in soilless medium, remained stable after 28 days of incubation.

Application of acibenzolar-*S*-methyl at 1.0 mg kg^{-1} slightly increased the populations of all PGPR strains while the fortification level of 10.0 mg kg^{-1} stimulated the bacterial populations to a greater degree after 35 days of incubation (Fig. 4). Insignificant differences in the number of colonies among all PGPR treatments compared to control were observed during metribuzin and napropamide applications. The data indicated that the number of PGPR colonies after 35 days of incubation in soil samples treated with one and ten times the recommended rates of the herbicides metribuzin and napropamide were not significantly different compared to the populations in untreated controls. Moreover, our results showed that the presence of 60 mg kg^{-1} propamocarb hydrochloride increased all bacterial populations after 35 days. The number of all PGPR colonies, at the end of the incubation time, was significantly increased to $2.71–3.12 \times 10^7$ CFU g^{-1} in soil samples supplemented with the recommended field dose of the compound propamocarb hydrochloride. Similarly, the addition of thiamethoxam caused a slight increase in the number of colonies at all PGPR strains tested. After 35 days of incubation, the number of colonies increased to $3.74–4.27 \times 10^7$ CFU g^{-1} and $4.07–4.52 \times 10^7$ CFU g^{-1} in soils treated with thiamethoxam at low and high fortification level, respectively, in comparison to the unspiked controls ($2.02–2.21 \times 10^7$ CFU g^{-1}). The stimulatory effect of soil applied insecticides on the populations of PGPR strains has

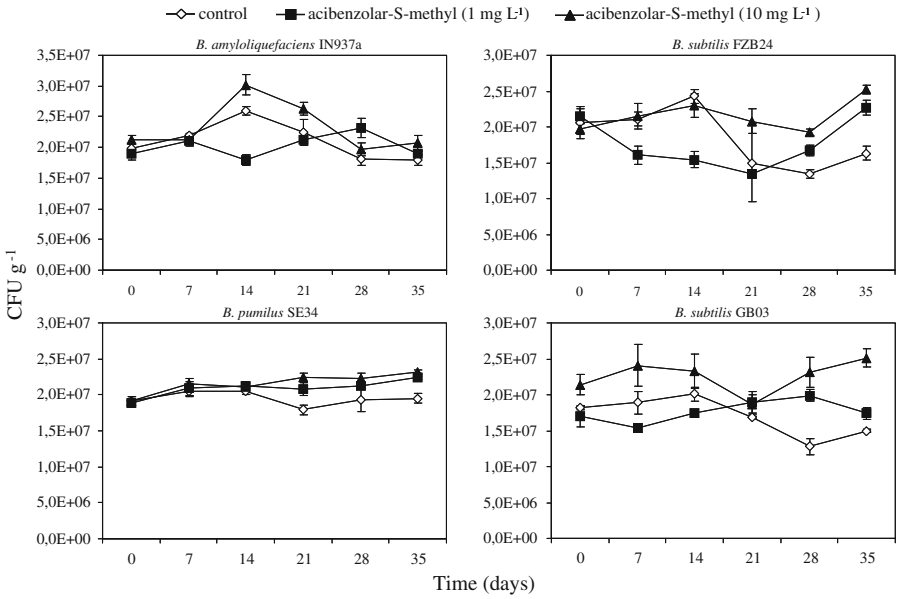

Fig. 4 Population survival of PGPR strains in sterile soil samples treated with acibenzolar-*S*-methyl at one and ten times the recommended rate of the compound, and untreated control. *Error bars* represent the standard deviation of the mean

been documented in a previous study by Martinez-Toledo et al. (1988). Also Lakshmi et al. (2009) have suggested, in a soil biodegradation experiment, that the populations of *Bacillus cereus*, *Klebsiella* sp., *Serratia marcescnes*, and *Pseudomonas aeruginosa* increased in soil samples contaminated with chlorpyrifos (50 mg kg^{-1}) during 30 days incubation period.

All experiments were conducted under controlled conditions (liquid medium and sterilized soil) in order to investigate the interactions of PGPR and soil applied pesticides and not to explore the potential of use PGPR for bioremediation of contaminated soils. The present study has demonstrated that the application of PGPR could significantly enhance the degradation of certain pesticides. Degradation of acibenzolar-*S*-methyl by all PGPR stains tested was 5.4–5.7 times faster than in non-inoculated medium. Moreover, degradation of acibenzolar-*S*-methyl proceeded rapidly in sterilized soil amended with all PGPR tested with $t_{1/2}$ values <16.4 days compared with >34.2 days in case of non-inoculated soil. For the remaining pesticides, PGPR stains were able to degrade 8–18, 9–11, 15–36 and 11–22% of metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam in liquid culture within 72-h incubation, respectively. PGPRs were unable to degrade metribuzin and napropamide in soil microcosm experiments. *B. amyloliquefaciens* IN937a and *B. pumilus* SE34 generally had greater and more prolonged impacts on degradation of propamocarb hydrochloride and thiamethoxam in soil. In all soil treatments, PGPR strains grew well with the varying concentrations of pesticides, and inhibitory effect of pesticides on the populations of PGPR strains was not observed. Even in many cases the addition of pesticides increased significantly the number of bacterial colonies.

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