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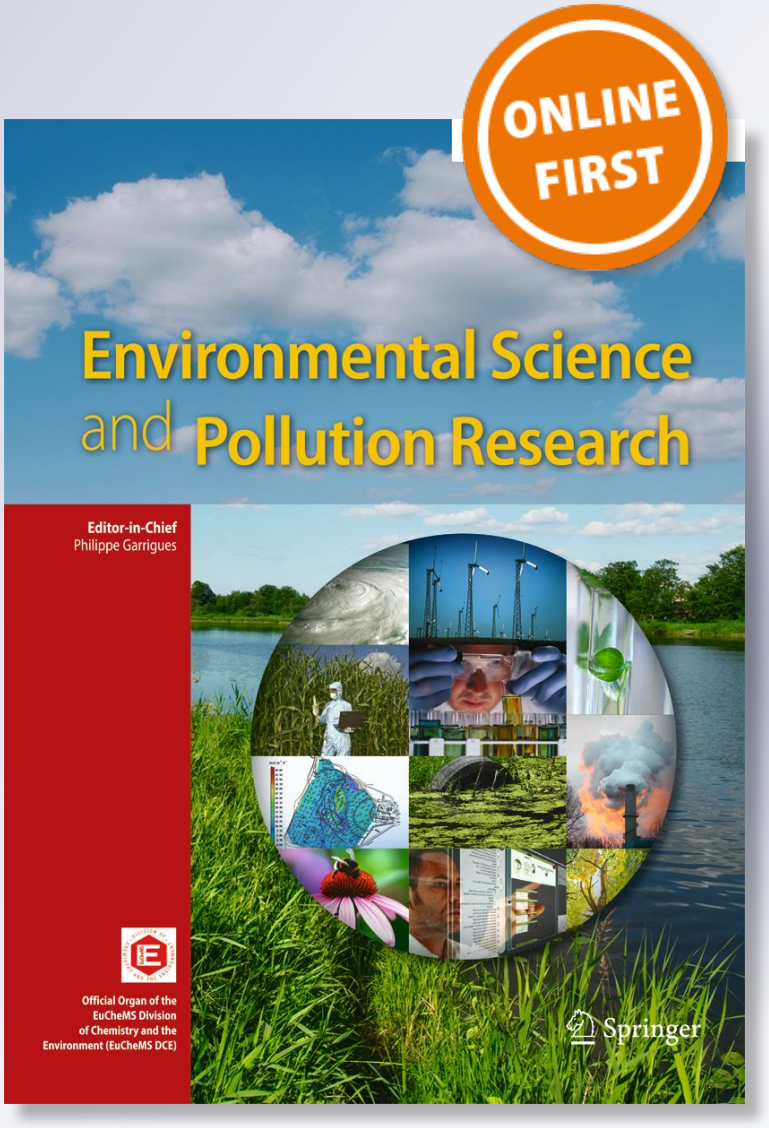

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RESEARCH ARTICLE

Brassica napus hairy roots and rhizobacteria for phenolic compounds removal

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Abstract Phenolic compounds are contaminants frequently found in water and soils. In the last years, some technologies such as phytoremediation have emerged to remediate contaminated sites. Plants alone are unable to completely degrade some pollutants; therefore, their association with rhizospheric bacteria has been proposed to increase phytoremediation potential, an approach called rhizoremediation. In this work, the ability of two rhizobacteria, *Burkholderia kururiensis* KP 23 and *Agrobacterium rhizogenes* LBA 9402, to tolerate and degrade phenolic compounds was evaluated. Both microorganisms were capable of tolerating high concentrations of phenol, 2,4-dichlorophenol (2,4-DCP), guaiacol, or pentachlorophenol (PCP), and degrading different concentrations of phenol and 2,4-DCP. Association of these bacterial strains with *B. napus* hairy roots, as model plant system, showed that the presence of both rhizospheric microorganisms, along with *B. napus* hairy roots, enhanced phenol degradation compared to *B. napus* hairy roots alone. These findings are interesting for future applications of these strains in phenol rhizoremediation processes, with whole plants, providing an efficient, economic, and sustainable remediation technology.

Keywords Removal · Rhizobacteria · Phenol · 2,4-DCP · Hairy roots · Interaction

Abbreviations

HR	Hairy roots
MM	Mineral medium
MS	Murashige–Skoog
MTC	Maximum tolerated concentration
2,4-DCP	2,4-Dichlorophenol

Introduction

Contamination of soil and water with phenolic compounds is a global problem. These hazardous compounds, such as phenol and 2,4-dichlorophenol (2,4-DCP), are frequently introduced in the environment through wastewater discharges from a variety of industries. They are also found in soils around chemical depots, manufactured gas, and wood-preserving industries. Due to the high toxicity of phenolic compounds, their presence in the ecosystem seriously affects living organisms (Whiteley and Bailey 2000; Arutchelvan et al. 2006). However, there are some microorganisms, such as bacteria, yeast, and algae, that can tolerate, degrade, and use these compounds as carbon and energy source (Van Schie and Young 2000). In this regard, biological degradation of phenolic compounds has been extensively investigated, and several studies have shown that phenol

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can be efficiently degraded by a wide variety of microorganisms (Leitao et al. 2007; Kurzbaum et al. 2010).

Recently, plant-associated bacteria, such as endophytic bacteria (non-pathogenic bacteria that reside within plant hosts) and rhizospheric bacteria (bacteria that live on and near plant roots), have been shown to contribute with degradation of toxic organic compounds, such as phenols. Besides contaminant removal, rhizospheric microbial communities can benefit plants by synthesizing compounds that protect them by decreasing plant stress hormone levels, delivering key plant nutrients and protecting them against plant pathogens (Gerhardt et al. 2009).

Among them, different genera such as *Burkholderia* and *Agrobacterium* have also been documented by several authors for their capability for phenolic compounds degradation (Schröder et al. 1997; Solomon et al. 1994; Kowalska et al. 1998). Regarding to *Burkholderia* species, it is well-known that they are very heterogeneous and occupy widely different ecological niches, including the rhizosphere of crop plants (Caballero-Mellado et al. 2007). Recently, many novel diazotrophic *Burkholderia* species, including legume nodule symbionts, as well as other plant-associated species have been found, but information about agronomic and environmental applications is scarce (Perin et al. 2006).

In the last years, plant tissue cultures (including cell and organ cultures) have been useful as experimental model systems to study the ability of plants to tolerate and remediate different phenolic compounds (Santos de Araujo et al. 2006). Among them, hairy roots (HR), obtained by infection of wounded plant tissues with *Agrobacterium rhizogenes*, are suitable for this purpose due to their rapid growth and high genetic stability. They are also an appropriate tool for plant physiology studies and could serve for plant–microorganisms association studies (Guillon et al. 2006). In this context, several HR derived from different plant species have been well characterized in our laboratory and tested for their ability for phenolic compound removal (González et al. 2006; Coniglio et al. 2008; Talano et al. 2010). Nevertheless, to our knowledge, there are few reports related to the use of HR for plant–microorganism association studies, which would be a suitable strategy in future applications of rhizoremediation processes (Able et al. 2003; Wu et al. 2007).

On the basis of these observations, the aims of the present investigation were: (a) to evaluate and compare phenolic compounds tolerance and degradation, by *Burkholderia kururiensis* KP 23 and *A. rhizogenes* LBA 9402, and (b) to analyze phenol removal efficiency by the association of these bacterial strains with *B. napus* (rapeseed) HR.

Materials and methods

Microorganisms and growth conditions

B. kururiensis KP 23, isolated from the rhizosphere of tomato (*Solanum lycopersicum*) plants (Caballero-Mellado et al. 2007), and *A. rhizogenes* LBA 9402 (collection strain) were used in this study. *B. kururiensis* KP 23 was maintained in TY medium (Beringer 1974) containing (g/L): triptone 5, yeast extract 3, $CaCl_2$ 0.65, and agar 13; whereas, *A. rhizogenes* was maintained in YMB medium (Hooykaas et al. 1977) containing (g/L): K_2HPO_4 0.5, $MgSO_4$ $7H_2O$ 2, NaCl 0.1, yeast extract 0.4, mannitol 10, and agar 13. Strains were sub-cultured every week and maintained at 28±2 °C.

Plant material

Rapeseed (*Brassica napus*) HR cultures were obtained as previously described (Agostini et al. 1997). They were subcultured every 30 d in Murashige–Skoog liquid medium (Murashige and Skoog 1962), enriched with vitamins and kept in an orbital shaker at 100 rpm and 25±2 °C, in darkness. HR cultures, at exponential phase of growth, were used for the experiments described below.

Bacterial tolerance assays to different phenolic compounds

For tolerance assays, both strains were spread on rich media agar plates (TY and YMB for *B. kururiensis* and *A. rhizogenes*, respectively), containing phenol (10–1,000 mg/L), 2,4-DCP (10–500 mg/L), PCP (20–500 mg/L), or guaiacol (500–1,500 mg/L). Subsequently, strains were spread on plates with agar mineral medium (MM1) containing (g/L): NaCl 2.5; KH_2PO_4 1.7, $MgSO_4$ $7H_2O$ 0.2, K_2HPO_4 4.3, NH_4SO_4 2.7, $CaCl_2$ 0.03 and supplemented with the same phenolic compounds concentrations, as were described above, to evaluate the use of these contaminants as sole carbon and energy source. Maximum tolerated concentration (MTC) was established as the highest tested concentration of the contaminant at which microorganisms could grow after 7–10 days.

Phenol and 2,4-DCP degradation by *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402

Degradation studies were carried out in different liquid mineral media (MM1, MM2, MM3) and MS medium. Mineral medium MM2 contained (g/L): K_2HPO_4 1.5; NaCl 0.5; KH_2PO_4 0.50; $CaCl_2$ 0.01; NH_4Cl 3.0; $FeSO_4$ 0.01. MM3 contained (g/L): KH_2PO_4 9; Na_2HPO_4 2; NH_4Cl 1; NaCl 2.5. Murashige–Skoog (MS) medium was also used for

bacterial growth but without vitamins and sucrose. All culture media were supplemented with phenol (200 and 500 mg/L) as sole carbon source, in order to find the optimum medium for phenol removal. In control conditions, phenol and 2,4-DCP were replaced by mannitol (1 g/L). Each strain was previously grown in TY liquid medium until late exponential phase and then, this culture was inoculated in Erlenmeyers flasks (to give an initial optical density (OD) of 0.05) containing 30 mL of the different media, with the contaminant.

Samples were incubated at 28 °C in an orbital shaker at 100–120 rpm. Growth was evaluated periodically by measurements of OD at 600 nm in a Beckman DU640 spectrophotometer. Phenol consumption was also evaluated in the samples as described below.

MM3 was selected for phenol and 2,4-DCP degradation studies. Different phenol (10–500 mg/L) and 2,4-DCP (10–50 mg/L) concentrations were added. Non-inoculated media, containing contaminants, were also included as controls.

Samples were taken for evaluation of growth and phenol or 2,4-DCP consumption. Each experiment was carried out in triplicate and average values are presented.

HR–microorganisms association studies

Root samples (100 mg) of *B. napus* HR were grown in MS medium during 14 days. At this time, HR cultures were inoculated with *B. kururiensis* KP 23 or *A. rhizogenes* LBA 9402. Filter-sterilized phenol solution was added to the cultures to reach a final concentration of 100 mg/L.

HR cultures plus phenol without microorganisms were used to evaluate phenol removal by HR. MS medium with phenol but without HR or microorganisms was used as control to evaluate possible losses of the contaminant by evaporation (abiotic control).

Samples were taken every day to evaluate phenol removal, following the methodology mentioned later. After 15 days, HR growth was determined by dry weight measurement. In all assays, results were expressed as removed phenol (%)/100 mg HR dry weight.

Phenol and 2,4-DCP determination

Phenol and 2,4-DCP were determined spectrophotometrically following a standard method described by Wagner and Nicell (2002). Aliquots of 100 μL of each sample (previously centrifuged) were mixed with 700 μL sodium bicarbonate (0.25 M, pH 8.4), 100 μL 4-aminoantipyrine (20.8 mM) and 100 μL potassium ferricyanide (83.4 mM). After 5 min, the absorbance of the colored compound formed was determined at 510 nm. Phenol and 2,4-DCP concentrations were calculated by comparison with a calibration curve.

Statistical analysis

Data were analyzed using ANOVA, followed by post hoc Dunnett test through Statgraphics Plus (version 7.1). In all cases, $p \leq 0.05$ was considered as statistically significant.

Results and discussion

Tolerance of *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402 to different phenolic compounds

Tolerance of both strains in rich (TY) and mineral (MM1) media containing different phenolic compounds concentrations was evaluated and the MTC for each contaminant was established (Table 1). In a rich medium, *B. kururiensis* KP 23 was able to grow in presence of 1,000 mg/L phenol or 1,500 mg/L guaiacol. At higher concentrations, growth inhibition was observed. When halogenated aromatic compounds such as 2,4-DCP or PCP were present in the medium, a lower tolerance was observed, since this strain grew only with 200 or 300 mg/L, respectively.

Regarding *A. rhizogenes*, similar results were observed. This strain also showed lower tolerance for 2,4-DCP and PCP than with phenol and guaiacol. However, *A. rhizogenes* presented a lower tolerance than *B. kururiensis*, for all phenolic compounds analyzed.

When tolerance was evaluated in mineral medium (MM1) supplemented with phenol, 2,4-DCP, PCP or guaiacol as sole carbon and energy source, both strains were able to grow with all the pollutants analyzed. However, tolerance was lower in mineral medium compared to that obtained in rich medium. In this sense, *B. kururiensis* KP 23 strain was

Table 1 MTC in rich (TY) and mineral (MM1) media by *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402

Medium	MTC (mg/L)		
	Phenolic compound	*B. kururiensis* KP 23	*A. rhizogenes* LBA 9402
Rich (TY)	Phenol	1,000	600
	Guaiacol	1,500	1,000
	2,4-DCP	200	70
	PCP	300	70
Mineral (MM1)	Phenol	700	500
	Guaiacol	1,000	750
	2,4-DCP	70	50
	PCP	200	20

able to tolerate higher concentrations of the different phenolic compounds than *A. rhizogenes* LBA 9402, which was in agreement with the results obtained in rich medium. It is important to note that the MTC of both microorganisms was lower for halogenated compounds (2,4-DCP and PCP) than for phenol or guaiacol, which could be associated with the higher toxicity of these compounds due to their chlorine substituents (Petroutsos et al. 2008).

Regarding to the relationship between soil bacteria and their response to toxic compounds, Muter et al. (2008) described at least four types of behavior: bacteria that are inhibited by the toxic, bacteria that are resistant, those which resist and degrade the toxic but do not grow and finally, bacteria that resist and degrade the toxic as well as use degradation products for biomass growth. As it could be seen, the analyzed strains were able to grow in a phenol-containing medium. However, this fact does not mean that the bacterial strain certainly degraded the contaminant. In this regard, it has been found that some bacteria possess an active efflux mechanism for monocyclic aromatic compounds (MACs) (Sharma et al. 2002) or polycyclic aromatic compounds (PACs) (Hearn et al. 2003) to with-stand their toxicity. Thus, we proceeded to analyze phenol and 2,4-DCP degradation.

Use of different culture media for phenol degradation

Environmental conditions are important factors that influence bacterial behaviour even in presence of toxic agents. Despite the difficulty to compare the effect of pure synthetic media with the conditions prevailing in real life situations (such as contaminated soil or water), the evaluation of different media composition and growth conditions for phenol degradation is necessary for the development of a bioremediation process (Ghanem et al. 2009).

In this work, different culture media were used to study their effects on phenol (200 and 500 mg/L) degradation, as well as the time required for this process.

Growth curves obtained in MM1, MM2, MM3 and MS media supplemented with phenol, for both strains, showed optical density values (OD 600 nm) which did not exceed 0.25-0.3, except for *B. kururiensis* that showed an increase in growth (OD 0.5 and 0.3) in MS medium, containing 200 and 500 mg/L phenol, respectively (data not shown). The results obtained for phenol degradation are shown in Table 2.

As it can be seen, the most efficient phenol removal process was obtained with *B. kururiensis* growing in MM3 medium. In contrast, *A. rhizogenes* was not able to completely remove phenol; however, in MM3 medium, it could remove 50 % of 500 mg/L phenol, reaching the highest removal efficiency. According to these results, phenol removal was more efficient in MM3 medium than in the other media, for both strains.

Table 2 Maximum phenol degradation (%) by *B. kururiensis* and *A. rhizogenes*, in different culture media, after 12–15 days

Medium	*B. kururiensis* KP 23		*A. rhizogenes* LBA 9402	
	Phenol 200 mg/L	Phenol 500 mg/L	Phenol 200 mg/L	Phenol 500 mg/L
MM1 (%)	50±5	10±2	7±2	12±2
MM2 (%)	15±3	15±4	25±3	10±2
MS (%)	25±5	15±3	0	5±1
MM3 (%)	100±10	100±15	27±2	50±5

Results are expresed as percentage

Establishment of appropriate and optimal conditions for phenol removal by microorganisms is relevant, since natural environments are subject to several fluctuations, like substrates availability, competition between different rhizospheric organisms, as well as changes in soil composition.

Effect of phenol and 2,4-DCP on *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402 growth and degradation capabilities in MM3 medium

Growth and phenol bioremediation of both strains were analyzed in MM3 medium. As it can be seen in Fig. 1 (A1, A2), the growth of both strains was considerably affected by phenol concentration from 10 to 500 mg/L, compared to control conditions, in which phenol was replaced for mannitol (1 g/L) as carbon source. Moreover, growth inhibition was concentration dependent.

Regarding phenol degradation, *B. kururiensis* was able to completely remove phenol and the time required for its degradation varied according to initial phenol concentration in the medium. In this sense, when concentrations varied between 10 and 500 mg/L, complete degradation of phenol occurred between 7 and 15 d, respectively (Fig. 1 (B1)). Phenol removal capability of this strain could be associated with the presence of mono-oxygenase aromatic genes detected in strains belonging to this genus by Caballero-Mellado et al. (2007).

In contrast, *A. rhizogenes* was only able to completely degrade 10 mg/L of phenol, and when higher phenol concentrations were used, a partial removal was observed (50 % of 500 mg/L).

Despite both strains were capable to remove phenol, the observed removal rate was low, since 7 and 15 days were necessary for a complete or partial phenol removal for *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402, respectively. In addition, the low growth reached during the exponential phase for both strains could be possible by the carbon source

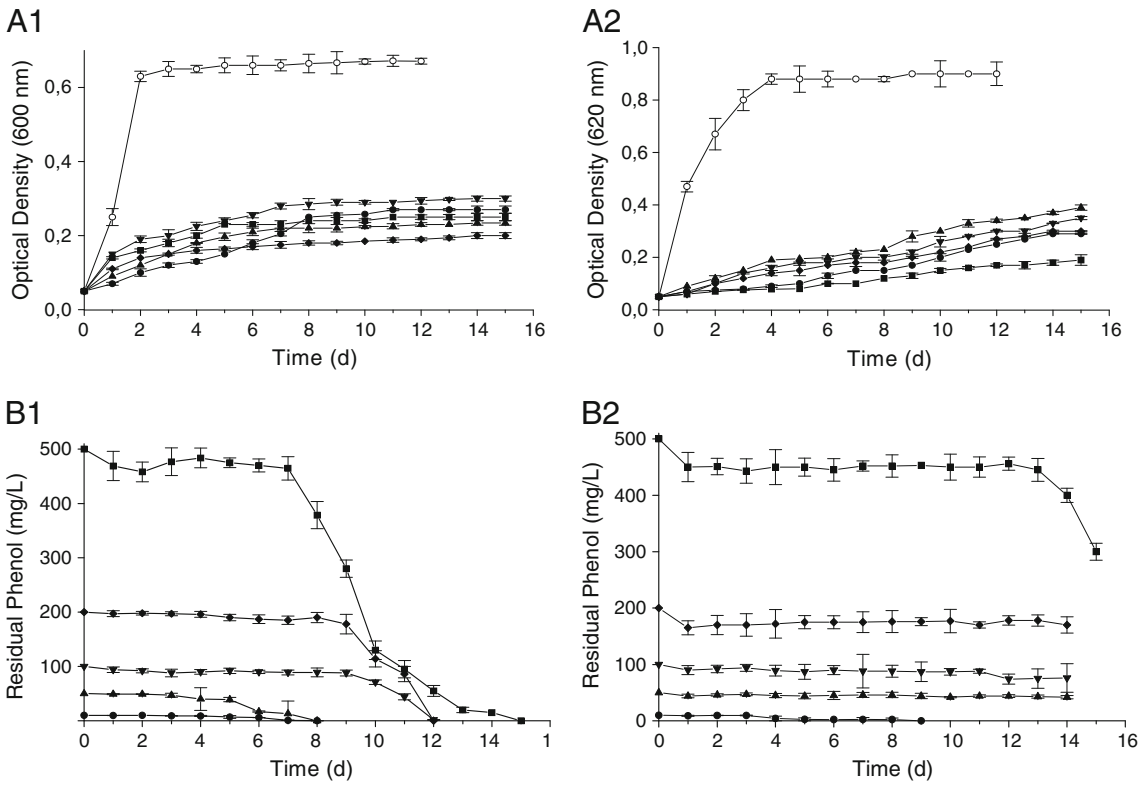

Fig. 1 Growth curves (*A1*, *A2*) and phenol degradation (*B1*, *B2*) of *B. kururiensis* KP 23 (*A1*, *B1*) and *A. rhizogenes* LBA 9402 (*A2*, *B2*) in MM3 medium with phenol: *circle* 0 mg/L, *filled circle* 10 mg/L, *filled triangle* 50 mg/L, *filled inverted triangle* 100 mg/L, *filled diamond* 200 mg/L, *filled square* 500 mg/L. The control curves were performed with mannitol (1 g/L) as carbon source

present in the inoculum (mannitol) and probably not by phenol consumption, since phenol degradation was coincident with stationary growth phase. It would indicate that phenol, at this phase of growth, could be incorporated and/or adsorbed by the microorganisms, but it could not be used for biomass production.

On the other hand, the effect of 2,4-DCP on *B. kururiensis* KP 23 and *A. rhizogenes* LBA 9402 growth and degradation ability was also evaluated. These studies were performed using 2,4-DCP concentrations of 10 and 50 mg/L, taking into account the MTC obtained for both microorganisms in mineral medium. As it is shown in Fig. 2 (A1, A2), the assayed concentrations produced high growth inhibition for both strains.

In addition, they were not able to remove 10 mg/L, probably due to an inappropriate C:N ratio present in the culture medium, which was consistent with the growth inhibition observed. However, when 2,4-DCP concentration was 50 mg/L, degradation of 40 % and 20 % was reached by *B. kururiensis* and *A. rhizogenes*, respectively. This finding may explain that the strains could not grow with 10 mg/L of this contaminant due to an insufficient C supply in the medium.

The low growth registered using 2,4-DCP as substrate, can be attributed to the toxicity and subsequent stress exerted by the contaminant. In this sense, it was described that halogenated compounds produce a toxic effect on microorganisms by disrupting energy transduction, either by uncoupling oxidative phosphorylation or by inhibiting electron transport (Petroutsos et al. 2008). Moreover, low biodegradation and prolonged periods of time to remove 2,4-DCP were frequently reported. For instance, Wang et al. (2000) observed that *Bacillus insolitus* was able to degrade similar 2,4-DCP concentrations than those used in the present work, after 16 d of incubation. In a similar way, Gallego et al. (2005) showed a reduced growth rate and prolonged latency phase of *Comamonas acidovorans* under 50 mg/L 2,4-DCP treatment.

Other factors that affect bacterial growth and degradation are the intrinsic physical and chemical properties of contaminants. Considering that 2,4-DCP shows a more complex structure than phenol, this fact could be the reason for the difficulty of its degradation, as it was pointed out by Annachhatre and Gheewala (1996).

Phenol degradation using *B. napus* HR–microorganisms

Plant tissue cultures are frequently used as experimental models for different studies. HR cultures exhibit all features of normal plant roots and they grow fast under

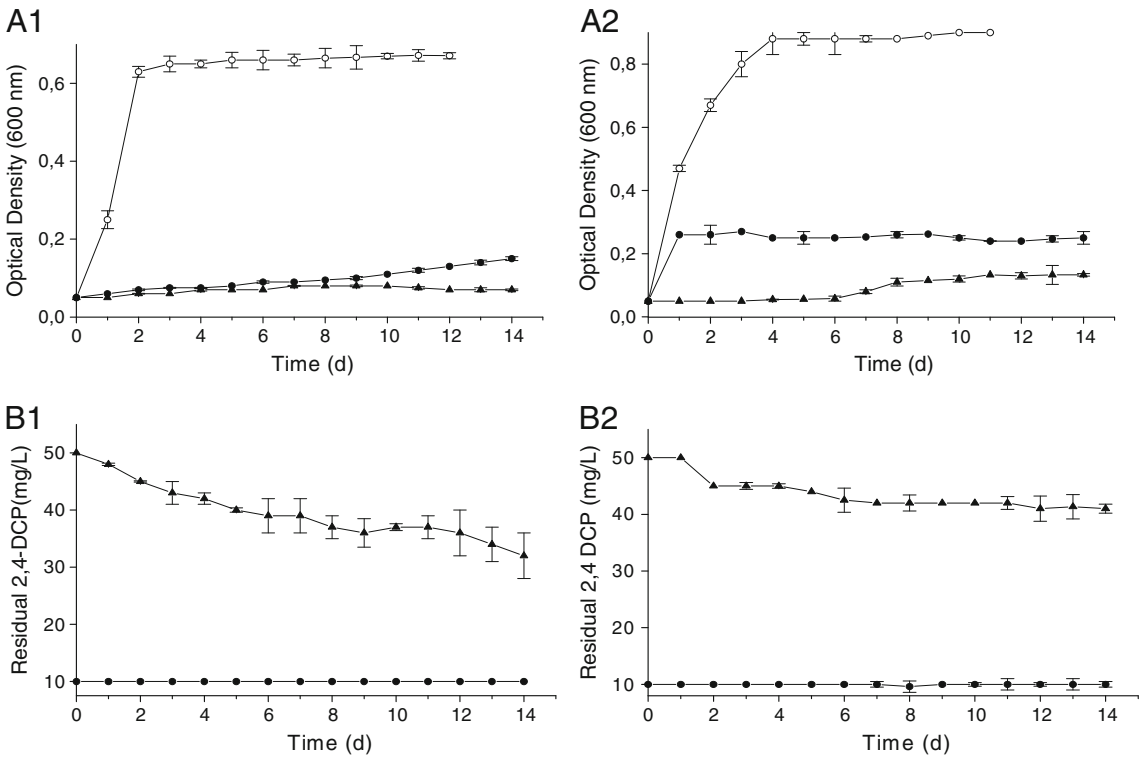

Fig. 2 Growth curves (*A1*, *A2*) and 2,4-DCP degradation (*B1*, *B2*) of *B. kururiensis* KP 23 (*A1*, *B1*) and *A. rhizogenes* LBA 9402 (*A2*, *B2*) in MM3 medium with 2,4-DCP: *circle* 0 mg/L, *filled circle* 10 mg/L, *filled triangle* 50 mg/L. The control curves were performed with mannitol (1 g/L) as carbon source

defined aseptic *in vitro* conditions (Macek 1989), thus allowing to differentiate between the plant metabolism itself and the effect of the complex interaction between plants and microbial communities in the rhizosphere (Macek et al. 1998). In addition, metabolism of many organic compounds has been addressed with the help of plant cells and tissue cultures. For instance, in previous work, it was established that HR derived from different plant species were capable to remove phenolic compounds (González et al. 2006; Coniglio et al. 2008). However, in the present work, we were interested in evaluating whether the association between HR and microorganisms could improve phenol degradation. For this, phenol removal assays were carried out using *B. napus* HR cultures inoculated with *B. kururiensis* or *A. rhizogenes*. The results obtained are shown in Fig. 3.

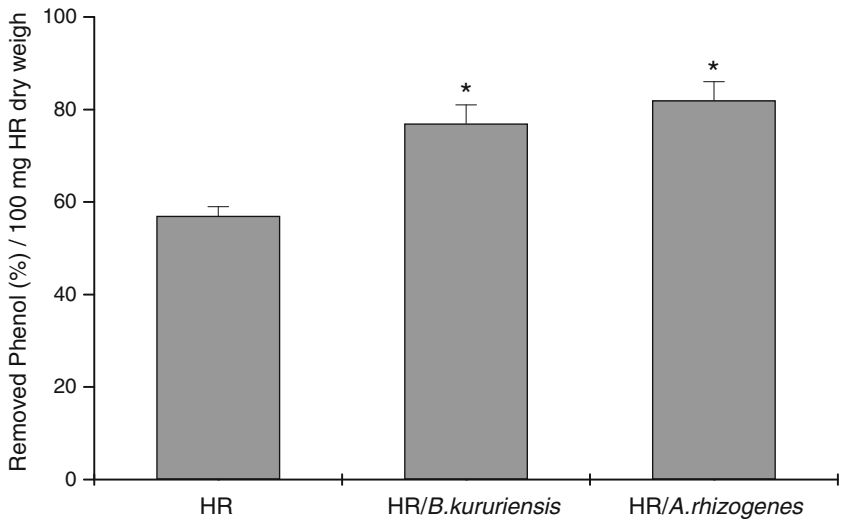

Fig. 3 Phenol removal obtained by the systems *B. napus* HR-*B. kururiensis* or *B. napus* HR-*A. rhizogenes* in MS medium, after 15 days. *B. napus* HR without inoculation were used as control. *Asterisks* indicate significant differences with the control. The differences between two treatments were not statistically significant ($p>0.05$)

It was observed that the presence of microorganisms in the medium had a positive effect on phenol removal. The co-inoculation of *B. kururiensis* was able to enhance phenol removal (34 %), whereas *A. rhizogenes* produced an increment of 40 % in removal efficiency, compared with HR without inoculation. However, removal efficiencies between treatments showed no statistically significant differences. Similarly, Narasimhan et al. (2003) showed an increase in PCBs degradation using *Arabidopsis* root-associated microbes, compared with the use of this plant alone. The authors suggested that these microorganisms can use plant secondary metabolites, such as phenylpropanoids as nutrients. In this sense, natural substances released by plant roots supply nutrients to microorganisms, which enhance their biological activities and stimulate the degradation of organic chemicals by inducing enzyme systems in the existing bacterial populations.

As it was demonstrated by several authors and also by the present results, a synergistic action of rhizospheric microorganisms and plants can lead to an increased removal and/or degradation of various xenobiotics, thus, providing useful basis for enhancing remediation of contaminated environments. In addition, the bioremediation process can also be stimulated by beneficial and growth-promoting microorganisms, especially rhizobacteria that colonize plant roots. Caballero-Mellado et al. (2007) described that *B. kururiensis* KP 23 was able to efficiently solubilize phosphate and to produce siderophores, which are two potential useful properties for plant growth promotion. Production of siderophores by plant growth-promoting rhizobacteria is considered to be important in the suppression of deleterious microorganisms and soil-borne plant pathogens (Siddiqui et al. 2005).Besides, in some cases, this appears to trigger induced systemic resistance (Compant et al. 2005). Moreover, Caballero-Mellado et al. (2007) demonstrated that maize inoculation with wild-type strains of *Burkholderia uname* promoted maize plant growth. Nevertheless, many bacteria capable of degrading certain organic pollutants cannot survive in the soil environment, while others cannot promote plant growth in uncontaminated soil. For example, *Pseudomonas fluorescens* P13 strain can facilitate plant growth, but could not degrade phenol and therefore cannot be used to remediate contaminated soils. Another strain, *Pseudomonas aeruginosa* SZH16, could not promote plant growth in uncontaminated soils, although it could facilitate plant growth, reducing phenol content in polluted soils (Yang et al. 2011).

Despite the fact that the present work is a first approach in plant–microorganism association studies for phenol remediation, the improved removal efficiencies found for both analyzed strains and HR, as model system, encourages us to test phenol remediation with whole plants in order to exploit their rhizoremediation potential.

Conclusion

B. kururiensis tolerated higher concentrations of phenol, 2,4-DCP, guaiacol and PCP than *A. rhizogenes*. These results are coincident with the higher capability of *B. kururiensis* to degrade phenol and 2,4-DCP than that observed for *A. rhizogenes*. Both strains were able to degrade pollutant concentrations that are usually found in contaminated environments.

Rhizoremediation studies using *B. napus* HR inoculated with *B. kururiensis* KP 23 or *A. rhizogenes* LBA 9402 showed that both microorganisms enhanced phenol degradation. These findings are interesting for future application of these strains in whole plant phenol rhizoremediation processes, considering that *B. kururiensis* KP23 is also able to solubilize phosphate and produce siderophores. In this context, with a global political shift towards green remediation technologies, the association between plants and microorganisms could provide an efficient, economic, and sustainable remediation technology.

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