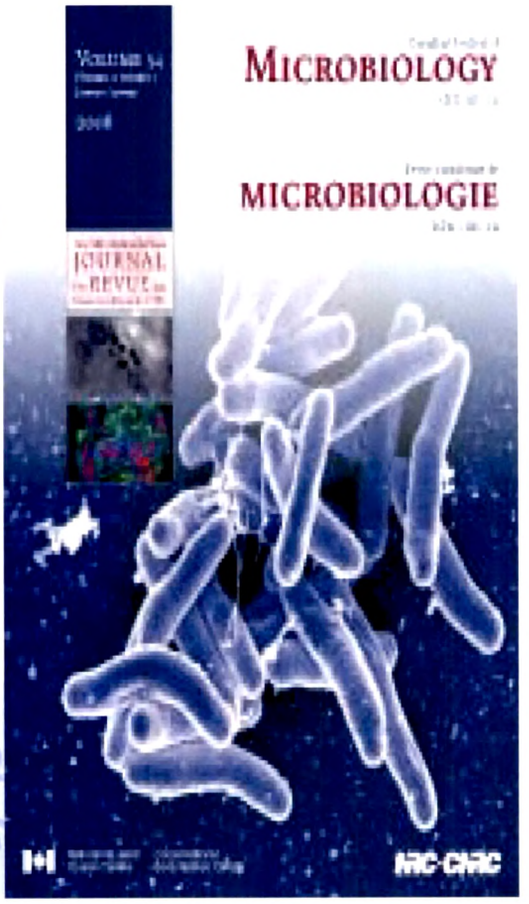
POSTERS, PRESENTATIONS AND PUBLICATIONS



Research paper published

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- Bee, H., Rupela, O. P., Sailasree J., Reddy, G., Sriveni, M., Naresh Kumar G. and **Sharma V.** "Solubilization of Rock Phosphate by Plant Growth Promoting Bacteria: Contrasting Performance in Two Different Soils". Presented in 6th International Plant Growth Promoting Rhizobacteria workshop held at Indian Institute of Spices research, Kozhikode (Calicut), Kerala during October 5-10, **2003**.
- **Vikas Sharma**, Sant, M., Archana, G. and Naresh Kumar, G. "Effect of different plasmids on the plant growth promoting properties of *Enterobacter asburiae* PSI3". Presented in 6th International Plant Growth Promoting Rhizobacteria workshop held at Indian Institute of Spices research, Kozhikode (Calicut), Kerala during October 5-10, **2003**.
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Substrate specificity of glucose dehydrogenase (GDH) of *Enterobacter asburiae* PSI3 and rock phosphate solubilization with GDH substrates as C sources

Vikas Sharma, Vikas Kumar, G. Archana, and G. Naresh Kumar

Abstract: *Enterobacter asburiae* PSI3 is a rhizospheric isolate that solubilizes mineral phosphates by the action of a phosphate starvation-inducible GDH (EC 1.1.5.2). We report here that GDH activity of this isolate shows broad substrate range, being able to act on mono and disaccharides. *Enterobacter asburiae* PSI3 was proficient at bringing about a drop in pH and solubilization of RP with the use of 75 mmol/L of each of the GDH substrate sugars tested as the sole C source. It liberated amounts of P ranging from 450 μmol/L (on arabinose) to 890 μmol/L (on glucose). When grown on a mixture of 7 GDH substrates at concentrations of 15 mmol/L each, the bacterium solubilized RP equivalent to 46% of the value when 75 mmol glucose/L was the C source. HPLC analysis of the culture supernatant under these conditions showed that the acidification of the media is primarily due to the production of organic acids. The significance of these results on the efficacy of *E. asburiae* PSI3 at solubilizing phosphates under rhizospheric conditions is discussed.

Key words: glucose dehydrogenase, substrate specificity, P solubilization, *Enterobacter asburiae* PSI3.

Résumé : *Enterobacter asburiae* PSI3 est un isolat de la rhizosphère qui solubilise les phosphates minéraux par l'action d'une D-glucose déshydrogénase (EC 1.1.5.2) (GDH) induite par la carence en phosphate. Nous rapportons dans cet article que l'activité GDH de cet isolat démontre un spectre de substrats élargi, capable d'agir sur des mono- et des disaccharides. *Enterobacter asburiae* PSI3 était compétent à diminuer le pH et à solubiliser le phosphate de roche (PR) en utilisant 75 mmol/L de chacun des sucres substrats de la GDH testés comme seule source de carbone et à libérer du P allant de 450 μmol/L (sur de l'arabinose) à 890 μmol/L (sur du glucose). Lorsque cultivés dans un mélange de 7 substrats de GDH à 15 mmol/L chaque, la bactérie a solubilisé l'équivalent de 46 % du PR solubilisé lorsque 75 mmol/L étaient utilisés comme sources de C. Une analyse par HPLC du surnageant de culture dans les conditions mentionnées ci-dessus a montré que l'acidification du milieu était principalement attribuable à la production d'acides organiques. La portée de ces résultats sur l'efficacité de *E. asburiae* PSI3 à solubiliser les phosphates dans les conditions retrouvées dans la rhizosphère est discutée.

Mots clés : glucose déshydrogénase, spécificité du substrat, stabilisation du P, *Enterobacter asburiae* PSI3.

[Traduit par la Rédaction]

Introduction

Rhizospheric microorganisms possessing the ability to solubilize mineral phosphates have been considered important for increasing P availability to the plants they associate with (Gyaneshwar et al. 2002; Kucey et al. 1989). Most PSMs dissolve poorly soluble soil phosphates by the acidification of the environment through secretion of organic acids (Kucey et al. 1989; Rodríguez and Fraga 1999; Sperber 1957). Among various PSMs studied, those secreting gluconic or 2-ketogluconic acids have been well-characterized, and acid secretion has been established as a basis of mineral phosphate solubilizing phenotype through gene cloning (Babu-Khan et al. 1995; Goldstein and Liu 1987; Kim et al. 1997, 2003; Liu et al. 1992) as well as by mutational analysis (Gyaneshwar et al. 1999). Gluconic acid production in PSMs is mediated by oxidation of glucose to gluconic acid by a PQQ-dependent GDH (EC 1.1.5.2). Depending on the bacterial species, gluconic acid may undergo additional oxidation by gluconate dehydrogenase resulting in production of 2-ketogluconic acid (Goldstein 1995).

GDH from many bacterial sources has been shown to possess broad substrate specificity and can catalyze the oxidation of several aldosugars to their respective lactones

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Abbreviations: GDH: D-glucose dehydrogenase; PQQ: pyrroloquinoline quinone; PSM: phosphate-solubilizing microorganism; RP: rock phosphate.

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(Goodwin and Anthony 1998). Two types of GDH enzymes have been identified to date: GDH A and GDH B, which differ in their subcellular localization and substrate specificity. GDH A, the more widespread isozyme, is membrane-bound and has been found in organisms such as *Acinetobacter calcoaceticus* (Cleton-Jansen et al. 1988), *Escherichia coli* (Ameyama et al. 1986), *Gluconobacter oxydans* (Ameyama et al. 1981), and *Pseudomonas fluorescens* (Matsushita et al. 1980), whereas GDH type B enzyme is soluble in nature and has been demonstrated only in *A. calcoaceticus* (Cleton-Jansen et al. 1989), although homologous sequences of uncharacterized proteins have been identified in the genomes of other bacteria (Oubrie et al. 1999).

Earlier work from this laboratory showed that PSMs that are proficient at solubilizing mineral phosphates in laboratory media are not necessarily able to liberate P from alkaline vertisols (Gyaneshwar et al. 1998). The high buffering capacity of alkaline vertisols and low amounts of organic acid secretion were held to be responsible for the inability to release P from soils. Incorporation of 100 mmol Tris–Cl buffer/L of pH 8.0 in the screening medium resulted in the isolation of *Enterobacter asburiae* strains, which secrete high amounts of gluconic acid and can release P from alkaline Indian soils (Gyaneshwar et al. 1999). One of these isolates, *E. asburiae* PSI3, isolated from the rhizosphere of pigeon pea (*Cajanus cajan*), secretes up to 55 mmol/L of gluconic acid/L when grown in the presence of 100 mmol glucose/L. The GDH of this organism has been demonstrated through mutational analysis to be responsible for the P-solubilization phenotype and is induced under phosphate starvation conditions (Gyaneshwar et al. 1999).

In this paper, we report the range of substrates acted upon by the GDH enzyme of *E. asburiae* PSI3 and the phosphate-solubilizing ability of this organism on different GDH substrates as C sources.

Materials and methods

Bacterial strains and culture conditions

Enterobacter asburiae PSI3, isolated from the rhizosphere of pigeon pea (Gyaneshwar et al. 1999), was used throughout these studies. Senegal RP used in the study contains (%) silica 5–6, P_2O_5 36–37, CaO 49–51, fluorine 3–4, insoluble acid 5–6, CO_2 1–2, Fe_2O_3 0.6–0.8, sulphate 0.3–0.6, and Al_2O_3 0.6–0.8. *Enterobacter asburiae* PSI3 was grown on minimal medium (Gyaneshwar et al. 1998) with RP as the sole P source in the presence of 100 mmol Tris–Cl/L, pH 8.0, and glucose, xylose, arabinose, galactose, mannose, maltose, or cellobiose as sole C sources at 75 mmol/L concentrations each, and in mixtures of these 7 sugars at concentrations of 15 and 10 mmol/L each. The culture was grown at 37 °C at 200 r·min^{-1} for different times until the pH was below 5.0. Aliquots were taken for measurement of pH and OD_{600} at 24-h intervals and centrifuged at 9200*g* for 5 min; the supernatant was used for P estimation (Ames 1964).

For organic acid analysis, *E. asburiae* PSI3 was grown on M9 minimal medium containing (for 1 L) Na_2HPO_4 30 g, KH_2PO_4 15 g, NH_4Cl 5 g, NaCl 2.5 g, and $CaCl_2$ 15 mg, supplemented with micronutrients consisting of (for 1 L) $FeSO_4·7H_2O$ 3.5 mg, $ZnSO_4·7H_2O$ 0.16 mg, $CuSO_4·5H_2O$ 0.08 mg, H_3BO_3 0.5 mg, $CaCl_2·2H_2O$ 0.03 mg, and $MnSO_4·4H_2O$ 0.4 mg. Different sugars were used at 100 mmol/L concentration as sole C sources. The organism was grown until the pH of the medium was reduced to 5.0, and the culture supernatant was used for HPLC analysis.

Measurement of GDH activity of *E. asburiae* PSI3

Cells were harvested (5000*g* for 10 min), washed with sterile saline, and resuspended in 50 mmol Tris–Cl/L, pH 8.75. Whole-cell suspensions were used as the source of enzyme in GDH assays carried out according to Matsushita and Ameyama (1982). To determine the GDH production on different sugars, *E. asburiae* PSI3 was grown on various sugars individually at 100 mmol/L concentration as sole C sources. Cells were harvested (5000*g* for 10 min) after 30 h, washed with sterile saline, and used for GDH assay. GDH activity was measured using 100 mmol glucose/L as substrate in the assay mixture. To determine the substrate specificity of the GDH enzyme, cells grown on glucose for 30 h were used for the enzyme assay with different sugars at 100 mmol/L concentration. These experiments were repeated 3 times. Results are expressed as means ± SD.

Analytical methods

Total protein was estimated using modified Lowry's method (Peterson 1979). Culture supernatant was used for pH and P estimation as described. P estimation was done by the ascorbate method (Ames 1964).

Organic acid analysis

Enterobacter asburiae PSI3 was grown on M9 minimal medium with different sugars at 100 mmol/L concentration as sole C sources. The organism was grown until the pH of the medium was reduced to 5.0. The culture supernatant was filtered using nylon-66 membrane filters of 0.22 µm pore size and subjected to HPLC with the following column specifications: HPLC (LaChrom, Merck, Darmstadt, Germany), reverse phase, ion pairing column (RP-18) column. The mobile phase consisted of 0.1% phosphoric acid at a flow rate of 1 mL/min. Detection was performed by a UV/VIS detector at 210 nm. The HPLC profile of the culture supernatants was analyzed by comparison with the elution profile of pure organic acids from HiMedia Laboratories Pvt Ltd., India.

Results

Substrate specificity and activity of GDH on different C sources

GDH activity of glucose-grown *E. asburiae* PSI3 was monitored with several sugars as substrates to determine its specificity (Table 1). GDH activity with galactose (110%) and xylose (94%) was comparable to activity with glucose (100%), whereas it was slightly low with maltose (75%), mannose (72%), and arabinose (66%). Cellobiose and 2-deoxyglucose showed 37% and 14% activity, respectively. When a mixture of 7 sugars, each at 15 mmol/L, was used as the substrate significant enzyme activity (46%) was found.

Table 1. Glucose dehydrogenase (GDH) activity of *Enterobacter asburiae* PSI3.

	GDH activity (U/mg protein)	
Sugar	Substrate[a]	C source[b]
D-Glucose	0.83±0.01 (100%)	0.38±0.02 (100%)
L-Arabinose	0.55±0.02 (66%)	0.18±0.02 (47%)
D-Xylose	0.78±0.01 (94%)	0.37±0.01 (97%)
D-Galactose	0.91±0.03 (110%)	0.31±0.02 (82%)
D-Maltose	0.62±0.01 (75%)	0.28±0.02 (74%)
D-Mannose	0.60±0.01 (72%)	0.26±0.01 (68%)
D-Cellobiose	0.31±0.03 (37%)	0.22±0.02 (58%)
Mixture[c]	0.38±0.01 (46%)	0.36±0.01 (95%)

Note: One unit of enzyme is defined as the amount that reduces 1 µmol of 2,6-dichlorophenol-indophenol/L per minute at 25 °C under specified conditions. Results are expressed as means ± SD of 3 independent experiments.

[a]Cells were grown on glucose as the sole C source, and different sugars were used as substrates in the GDH assay to measure their rates of direct oxidation.

[b]Cells were grown on the various sugars as C sources, and glucose was used as a substrate to measure GDH activity.

[c]Mixture indicates 15 mmol/L of each of the 7 sugars independently tested.

GDH activity of *E. asburiae* PSI3 was also monitored after growth on different sugars (Table 1) using 100 mmol glucose/L as the substrate. GDH activity on xylose (97%) and on galactose (82%) was similar to that on glucose (100%). GDH activity was lower on other sugars, such as maltose (74%), mannose (68%), cellobiose (58%), and arabinose (47%). When *E. asburiae* PSI3 was grown on a mixture of 7 sugars at 15 mmol/L each, GDH activity was found to be 95% of that when grown on 100 mmol glucose/L.

Solubilization of RP by *E. asburiae* PSI3 on different sugars

Enterobacter asburiae PSI3, when grown on RP as the sole P source in the presence of 100 mmol Tris–Cl/L, pH 8.0, acidified the medium to pH less than 5.0 with glucose, xylose, arabinose, galactose, mannose, maltose, or cellobiose as individual C sources at a 75 mmol/L concentration (Table 2). On sucrose and fructose, *E. asburiae* PSI3 did not show RP solubilization, although these sugars could be used as C sources for growth in the presence of soluble P (data not shown). The time required for acidification of the medium varied from 30 h in the case of cellobiose to 96 h for arabinose. Growth ranged from 0.22 OD_{600} in the case of glucose to 0.78 OD_{600} on mannose. The amount of soluble P varied from 450 µmol/L for arabinose to 890 µmol/L for glucose. A mixture of all 7 sugars, at 15 mmol/L each, in medium buffered with 100 mmol Tris–Cl/L, pH 8.0, resulted in the acidification of medium to pH less than 5.0 within 96 h, and the amount of P solubilized was 410 µmol/L. A mixture of 10 mmol/L of each sugar could bring about acidification of the medium to pH 6.23 in 96 h, and P released in the supernatant was found to be 100 µmol/L.

Organic acid analysis

To determine the nature of organic acids secreted by *E. asburiae* PSI3 while growing on different sugars, the culture supernatants were collected after the pH drop and analyzed by HPLC. Culture supernatants showed major peaks at the following retention times (in minutes) with different sugars as C sources: 2.40 on glucose, 2.36 and 3.10 on arabinose, 3.40 on galactose, 3.40 on mannose, 3.35 on xylose, 3.44 on maltose, and 2.66 on cellobiose. Of these, the peak obtained on glucose-containing medium with a retention time of 2.40 min corresponded to pure gluconic acid. None of the other peaks matched with the following standard acids, whose retention times were (in minutes) 2.26 for oxalic acid, 4.27 for lactic acid, and 4.54 for acetic acid.

Discussion

Gluconic acid production as a result of the activity of periplasmic or membrane-bound GDH is one of the best studied mechanisms by which PSMs liberate P from poorly soluble mineral phosphates (Goldstein 1995). Although GDH enzymes from various bacteria are well-characterized in terms of kinetic properties and mechanisms (Goodwin and Anthony 1998; Elias et al. 2004), substrate range characteristics of GDH from P-solubilizing isolates have not been previously reported. The substrate specificity of GDH from P-solubilizing *E. asburiae* PSI3 presented in this paper is compared with that of reported GDH enzymes in Table 3. The GDH enzyme of *E. asburiae* PSI3 shows broad substrate range, including hexoses, pentoses, as well as disaccharides. The results are in agreement with the general opinion that the enzymatic characteristics of bacterial GDHs vary depending on the specific bacterial sources (Yoshida et al. 1999). *Enterobacter asburiae* PSI3 GDH enzyme differs from other GDH enzymes by the ability to act on both mono and disaccharides.

The GDH activity of *E. asburiae* PSI3 varied, depending on the nature of the sugars present in the medium, the greatest activity being present on glucose and xylose. The GDH enzyme of this organism is present in cells grown on several sugars as C sources (including those that are not GDH substrates, such as sucrose and fructose, which could be used by *E. asburiae* as C sources for growth but not for RP solubilization, data not shown). This indicates the constitutive nature of the enzyme and is in contrast to the *E. coli* enzyme, whose levels are negatively regulated by glucose through cAMP (Izu et al. 2002; Yamada et al. 1993). Bacteria are known to utilize sugars that serve as GDH substrates by different pathways. High GDH activity was observed when *E. asburiae* PSI3 was grown on glucose or xylose. These sugars are utilized mainly through glycolysis and pentose phosphate pathways, respectively. Mannose, utilized by the glycolytic pathway, showed low GDH activity. Thus, levels of GDH of *E. asburiae* PSI3, when grown on a particular sugar, do not seem to show a relation with the pathway of sugar metabolism.

A novel aspect of this study is the correlation between the broad substrate specificity of the GDH enzyme and the manifestation of the P-solubilization phenotype on several C sources. Previous studies from this and other laboratories on gluconic-acid-producing PSMs were primarily concerned with the P-solubilization phenotype under conditions in which glucose was the sole C source. When *E. asburiae*

Table 2. Solubilization of RP by *E. asburiae* PSI3 on different C sources.

C source (mmol/L)	Time taken for pH drop (h)	pH	OD_{600}[a]	Amount of P released (mmol/L)
D-Glucose (75)	60±0	4.42±0.10	0.22±0.06	0.89±0.06 (100%)
D-Cellobiose (75)	30±0	4.88±0.11	0.54±0.05	0.78±0.03 (88%)
D-Xylose (75)	68±6	4.64±0.20	0.24±0.04	0.75±0.02 (84%)
D-Mannose (75)	46±3	4.65±0.21	0.78±0.08	0.68±0.06 (76%)
D-Galactose (75)	50±3	4.90±0.23	0.73±0.03	0.60±0.10 (67%)
D-Maltose (75)	40±6	4.35±0.04	0.66±0.06	0.47±0.15 (53%)
L-Arabinose (75)	96±0	4.86±0.09	0.24±0.00	0.45±0.03 (50%)
Mixture 15 each	40±4	4.68±0.25	0.43±0.07	0.41±0.15 (46%)
Mixture 10 each	96±5	6.23±0.21	0.70±0.07	0.10±0.13 (11%)

Note: Results are expressed as means ± SD of 3 independent experiments.
[a]The initial OD_{600} was approximately 0.05.

Table 3. Comparison of the substrate specificity of GDH enzymes from various bacteria.

Substrate	*Escherichia coli* GDH A (Ameyama 1986)	*Acinetobacter calcoaceticus* GDH A (Cleton-Jansen 1988)	*Acinetobacter calcoaceticus* GDH B (Cleton-Jansen 1989)	*Pseudomonas fluorescens* GDH A (Matsushita 1980)	*Gluconobacter oxydans* GDH A (Ameyama 1981)	*Enterobacter asburiae* PSI3 (This work)
D-Glucose	100%	100%	100%	100%	100%	100%
D-Allose	n.t. (++)[a]	n.t.	66%	n.t.	n.t.	n.t.
L-Arabinose	n.t. (++)[a]	95%	35%	3%	n.t.	66%
2-Deoxyglucose	n.t. (++)[a]	100%	4%	n.t.	n.t.	14%
D-Fucose	91%	n.t.	n.t.	n.t.	n.t.	n.t.
D-Galactose	41%	90%	18%	7%	0%	110%
D-Mannose	38%	n.t.	4%	9%	0%	72%
L-Rhamnose	20%	0%	n.t.	8%	0%	n.t.
D-Ribose	0% (+)[a]	80%	1%	0%	0%	n.t.
D-Xylose	54%	100%	20%	13%	0%	94%
Cellobiose	n.t.	n.t.	70%	n.t.	n.t.	37%
Lactose	n.t.	0%	65%	n.t.	n.t.	n.t.
Maltose	10%	0%	90%	3%	5%	75%
Melibiose	n.t. (++)[a]	n.t.	10%	n.t.	n.t.	n.t.

Note: n.t., not tested.
[a]Symbols in parentheses denote the results from Cozier et al. (1999). (+), V_{max} approx. < 300 μmol/min per mg; (++), V_{max} approx. > 400 μmol/min per mg.

PSI3 was grown with 75 mmol/L of each of the other GDH substrates as the sole C source, pH drop was observed under strongly buffered conditions, and P_i was liberated. Substitution of KH_2PO_4 as the P source in place of RP allowed enhanced growth (data not shown), indicating that growth on the buffered RP medium was probably strongly dependent on P solubilization. Upon use of 50 mmol/L of individual sugars as C sources under similar conditions, the pH did not drop to below 5, and P solubilization was insignificant up to 96 h (data not shown). HPLC analysis revealed the absence of secreted fermentation products, such as acetate and lactate, and the acidification of the medium was possibly due to the conversion of different sugars to their respective aldonic acids by GDH-mediated direct oxidation. *Enterobacter asburiae* PSI3 grown on sucrose and fructose did not solublize RP on plates as well as in the liquid medium. The results suggest that GDH-mediated acidification is the main mechanism of P solubilization by *E. asburiae* PSI3, and aldonic acids generated by the action of GDH on various sugars can bring about P solubilization. The role of GDH *E. asburiae* PSI3 in P solubilization has been demonstrated by the isolation of GDH mutants with a defective P-solubilization phenotype (Gyaneshwar et al. 1999).

Reports that demonstrate the use of various C sources for determining optimum conditions for P solubilization generally use each sugar individually at high concentrations (Cerezine et al. 1988). An important aspect of this work is the demonstration of P solubilization with a mixture of sugars, each at a concentration much lower than that generally used and too low to support P solubilization independently. When all the 7 GDH substrates were supplied as a mixture at 15 mmol/L each (the combined sugar concentration was 105 mmol/L), substantial pH drop and P solubilization were observed. Even when individual sugars in the mixture were at a concentration of 10 mmol/L each, 100 μmol P/L was released. This indicates the concurrent action of GDH on different sugars to produce sufficient acid to bring about P release.

The availability of adequate and appropriate C sources in the soil is known to be one of the major limiting factors for

the survival, growth, and functioning of microorganisms in soils. Using the *Pfic* reporter gene system in *P. fluorescens*, C source has been demonstrated to be limited in soils (Koch et al. 2001). However, rhizospheric soil is richer in C source since plants secrete nearly 5%–21% of photosynthetically fixed carbon through root exudates (Walker et al. 2003). With the use of *inaZ* reporter under *scrY* promoter, sucrose has been shown to be present at 100-fold higher concentration in the rhizosphere than in the bulk soil (Jaeger III et al. 1999). Generally, root exudates contain several sugars at low concentrations (Matsushita and Ameyama 1982). It has been suggested that an organism that is capable of using a variety of C sources for its bioenergetics could have an advantage over competitors that use limited C sources in natural environments (Goodwin and Anthony 1998). The versatile use of various C sources for P solubilization by *E. asburiae* PSI3 could thus be significant from the perspective of field performance.

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