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Short communication

Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria

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Abstract

Seven isolates of orchid-associated bacteria (OAB) belonging to five species were tested for their effect on mycorrhiza-assisted germination of the terrestrial orchid *Pterostylis vittata*. Hormone standards were also tested to evaluate their potential roles in the germination and development of the orchid. Strains of *Pseudomonas putida*, *Xanthomonas maltophilia* and *Bacillus cereus* promoted symbiotic germination, whereas certain strains of *P. putida* and an *Arthrobacter* species reduced it. Symbiotic germination was enhanced by IAA, inhibited by gibberellic acid and suppressed by kinetin. Each species of OAB produced IAA, although the conditions of growth affected the production of the auxin. IAA was not produced by the mycorrhizal fungus from *P. vittata* under the test conditions. Enhancement of symbiotic germination development may have resulted either from the production of IAA by the OAB and/or by the induction of endogenous hormones in the orchid by the metabolites of the bacteria and/or mycorrhizal fungus.

Introduction

In a previous study, bacteria were isolated from the mycorrhizal tissues of Western Australian orchids (Wilkinson et al., 1989). These isolates were subsequently identified (Wilkinson et al., 1993) using the fatty acid analysis method (Hewlett-Packard, 1987; Sasser, 1990). These orchid-associated bacteria (OAB) follow a seasonal pattern of abundance that differs between orchid genera, especially on the basis of the morphology of fungus-infected tissue (Wilkinson et al., 1989). Twenty-nine species of OAB were identified, belonging mainly to the genera *Pseudomonas* and *Bacillus*. (Wilkinson et al., 1993). Some strains of OAB are also capable of promoting the symbiotic germination of *Pterostylis vittata* Lindley (Wilkinson et al., 1989). The production of plant growth substances in the rhizosphere of plants has often been suggested as a mechanism for plant growth promotion by particular strains of soil bacteria (Barea et al., 1976; Cacciari et al., 1989; Tien et al., 1979). This paper examines the effect of selected OAB strains of different genera on symbiotic germination of *P. vittata*, the influence of hormone standards on symbiotic germination and the potential of the same OAB strains and a mycorrhizal fungus from *P. vittata* to produce indoleacetic acid in pure culture.

Materials and methods

Bacterial enhancement of symbiotic germination

Seven OAB isolates were tested for their influence on the symbiotic germination of *Pterostylis vittata* seed collected from wild sources in bushland sites in Kings Park and Botanic Garden, Perth, Australia. The bacteria were grown in tryptic soy broth (TSB; Sigma Chemical Co., St. Louis) at 25 °C in darkness for 24–36 hrs after which 10-fold dilutions were made in sterile deionized water to yield approximately 10^3 cfu mL^{-1}. 0.1 mL of this dilution for each isolate was added to the surface of oatmeal agar within Petri plates prior to introduction of orchid seed and the inoculum of the mycorrhizal fungus (see Ramsay et al., 1986). Orchid seed was surface sterilised for 5 min in 1% sodium hypochlorite containing tween-80. Seed was then rinsed in two changes of sterile water and smeared carefully onto strips of sterile Whatman No. 1 filter paper. Following sowing of seed, inoculum (approx. 40 mm^3) of the mycorrhizal fungus from the edge of an actively growing culture on potato dextrose agar was added to each plate.

Five replicates were prepared per treatment and the plates were incubated as shown in Table 1.

The total number of germinants were counted for each treatment at 10 × magnification (Ramsay et al., 1986). The treatments were analysed for significance by fitting a log-linear regression model with Poisson errors using the program 'GLIM' (Healy, 1988) and the results presented as number of seedlings.

Orchid seeds are minute, and it is difficult to estimate their exact numbers on agar media. To account for this, an estimation of the total number of seeds per plate was included as a factor in the analysis, whereby 1 = high numbers (> 5000 seeds), 2 = medium numbers (3000–5000) and 3 = low numbers (< 3000). A significantly lower number of protocorms were recruited from a seed number category of 3 than from a seed number category of 1 (χ^2 2.5%) as a result of the lower seed sowing density.

Effect of hormones on symbiotic germination

A trial was set up to determine the effect of hormone standards on the symbiotic germination of *P. vittata*. This was arranged as a factorial design experiment, incorporating 3 hormones (IAA, gibberellic acid (GA_3) and kinetin; Sigma Chemical Co.) at 4 concentration levels (0, 1, 5 and 10 mg L^{-1}). The filter-sterilised (Millipore 0.2 μm) hormone standards were added to cooled autoclaved oatmeal agar in 100 mL glass bottles just prior to setting. The bottles were shaken thoroughly and poured immediately to facilitate rapid cooling and setting. Orchid seed sterilisation and sowing, incorporation of mycorrhizal fungus and incubation conditions were as detailed above and in Table 1. This trial was scored and analysed as described above.

Production of IAA by OAB strains and the mycorrhizal fungus from P. vittata

The mycorrhizal fungus from *P. vittata* and selected OAB isolates picked at random were tested for their ability to produce IAA in pure culture. Bacterial isolates were grown in 200 mL TSB at 25 and/or 30 °C for 3 and 6 days prior to extraction. Similarly, the mycorrhizal fungus

Table 1. Incubation conditions employed in symbiotic germination trials of *Pterostylis vittata* seed incorporating isolates of orchid-associated bacteria (OAB) and hormone standards

OAB isolate numbers	Incubation conditions
Hormone standards *P. putida* (isolates 1 and 2) *B. sphaericus* (isolate 5)	30 days in darkness then 25 days under lights at 18 °C
P. putida (isolate 3) *X. maltophilia* (isolate 4) *B. cereus* (isolate 6) *Arthrobacter* sp. (isolate 7)	30 days in darkness at 25 °C then 20 days under lights at 18 °C

from *P. vittata* was grown for 30 days in darkness in 200 mL of the medium of Barroso et al. (1986). The extraction procedure used in this study was similar to that described by Tien et al. (1979). After incubation, the cell-free culture filtrates were acidified to pH 2.8–2.9 and extracted 3 times with 50 mL ethyl acetate. The ethyl acetate fractions were combined and evaporated under vacuum at 30 °C to near dryness in a rotary evaporator (Buchi, Rotavapor-R, Switzerland). The moist residue was redissolved in 3 mL methanol and chromatographed on 0.25 mm thick silica gel thin-layer chromatography (TLC) plates (Merck, Germany). The solvent system was chloroform: methanol: acetic acid (85:15:1) and the developing distance was 10 cm. After development, the plates were sprayed with 2% Ehrlich reagent to detect possible auxin compounds (Bentley, 1962). OAB and fungal culture extracts were co-chromatographed with an IAA standard and a control which was an extract of the original media in which the bacteria or fungus had not been inoculated. One OAB extract (from isolate 5, a *Bacillus sphaericus* Meyer and Neide strain) was also examined by mass spectrometry since TLC of this extract provided good evidence for the presence of IAA. This extract was purified on an 0.5-mm thick silica gel preparative TLC plate developed in chloroform: methanol: acetic acid (85:15:1) as above. The zone of silica gel corresponding to the Rf of the IAA standard was removed from the plate and eluted for 30 min in 20 mL ethyl acetate. After filtering, the eluate was evaporated under vacuum as described above and the moist residue was redissolved in 0.5 mL ethyl acetate. The sample was analyzed by direct-insertion mass spectrometry using a Hewlett-Packard 5986 MS instrument operating at 30 eV, sample probe temperature 120 °C. A mass spectrum of an authentic sample of IAA was obtained under identical conditions. The ions at m/z 175, 130, 131, 129, 103, 77 and 44 were used for a comparison of the two spectra.

Results

Some orchid-associated bacteria were capable of promoting the symbiotic germination of *P. vittata* seed (Fig. 1). In some instances, the magnitude of promotion was substantial (up to 200%), while some isolates had no effect and others significantly inhibited symbiotic germination (Fig. 1). Promotion or inhibition was not consistent to any group of bacteria, since bacteria of the same species (e.g., isolates 2 and 3 belonging to *P. putida* (Trev.) Migula) induced very different responses (Fig. 1). Furthermore, only treatments that were subjected to the change in temperature (from 25 to 18 °C) after the commencement of germination (isolates 3,4, 6 and 7) resulted in significant promotion of germination (Fig. 1).

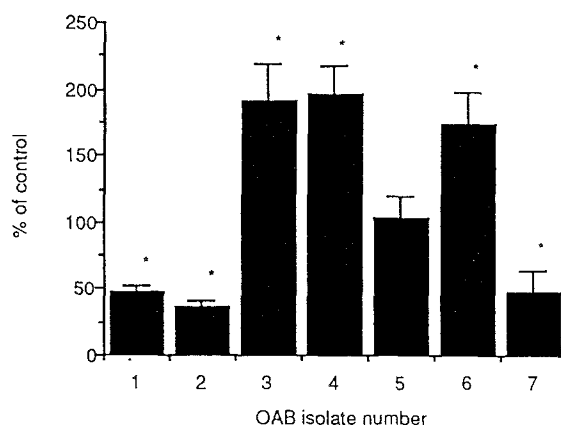

Fig. 1. The influence of orchid-associated bacteria (OAB) strains on the symbiotic germination of *Pterostylis vittata*. *Significantly different from control ($p < 0.05$). OAB isolates 1, 2 and 3 belonged to *Pseudomonas putida*; 4 to *Xanthomonas maltophilia*; 5 to *Bacillus sphaericus*; 6 to *Bacillus cereus*; and 7 to *Arthrobacter* sp.

Symbiotic germination of *P. vittata* seed appeared to be very sensitive to the presence of plant growth regulators (Table 2). IAA promoted germination at 5 and 10 mg L^{-1} (Table 2). In contrast all levels (≥ 1 mg L^{-1}) of GA_3 were inhibitory even in combination with IAA (Table 2). Kinetin completely suppressed germination at all levels tested (≥ 1 mg L^{-1}).

Five of the seven OAB isolates tested produced IAA in pure culture (Table 3). Extracts obtained from the cell-free culture filtrates of these, on TLC analysis, showed the presence of a compound having the same characteristics as those of a standard sample of IAA. To confirm the identity of the compound, the extract from *Bacillus sphaericus* (isolate 5) was separated by preparative TLC and the material corresponding in Rf to IAA was isolated. A mass spectrum of

Table 2. The effect of the addition of indole-acetic acid (IAA) and gibberellic acid (GA_3) on the mean number of symbiotically germinated seedlings of *Pterostylis vittata*. Values for treatments incorporating kinetin are not presented since germination did not occur in these treatments. Values are mean counts of number of seedlings from 5 replicates. Standard errors are presented in parentheses

IAA (mg L^{-1})	GA_3(mg L^{-1}) (Number of seedlings)			
	0	1	5	10
0	71.4 (7.8)[a]	0	37.2 (6.8)	37.2 (6.7)
1	79.4 (16.6)[b]	55.8 (9.5)	35.0 (6.7)	33.4 (6.0)
5	129.8 (11.6)	41.2 (4.6)	37.0 (8.4)	43.6 (4.5)
10	116.2 (29.6)	42.0 (4.4)	34.0 (9.2)	63.8 (10.4)

[a] Control treatment (i.e. no hormones).
[b] Not significantly different from control ($p < 0.05$). All other values are significantly different ($p < 0.05$) from the control.

Table 3. Detection of IAA in culture filtrate of selected strains of orchid-associated bacteria by thin-layer chromatography

Isolate number	Identification	Incubation time (days)	Incubation temp. (°C)	IAA detected
1	*Pseudomonas putida*	6	25	−
2	*P. putida*	6	25	−
3	*P. putida*	6	25	+
4	*Xanthomonas maltophilia*	3	25	−
		6	25	−
		6	30	+
5	*Bacillus sphaericus*	3	30	+
		6	30	+
		6	25	+
6	*B. cereus*	3	25	−
		6	25	+
7	*Arthrobacter* sp.	6	25	+

this sample was similar to that obtained from an authentic sample of IAA. Strains of *Xanthomonas maltophilia*, *Bacillus sphaericus*, *B. cereus*, *Arthrobacter* sp. and *Pseudomonas putida* produced IAA while two strains of *P. putida* (isolates 1 and 2) failed to produced detectable levels of the auxin. IAA was not detected in the culture filtrates of the mycorrhizal fungus under the test conditions.

The production of IAA by the bacterial isolates depended on the temperature and period of incubation. *Xanthomonas maltophilia* (isolate 4) for example, produced detectable IAA at 30 °C, but not at 25 °C (Table 3).

Discussion

Although some isolates promoted symbiotic germination, effects were variable from one test series to the next and appear to be affected by changes in environmental conditions (Wilkinson et al., 1990). The observed responses may be due to the involvement of beneficial or deleterious substances produced by the bacteria in response to exudates released by the germinating seeds on the agar medium. Enhanced germination and seedling growth in the presence of hormones has been previously recorded for orchids, but the requirement for these substances are not considered to be absolute (Arditti et al., 1990) and results have often been variable and inconclusive (Arditti and Ernst, 1984). However, it appears that this was concluded from studying asymbiotic germination, with the assumption that the requirements for symbiotic germination are identical. This is the first record of the response of a symbiotically germinating orchid to applied IAA. The production of IAA in pure culture by OAB in our study confirms earlier reports with soil bacteria (Barea et al., 1976; Cacciari et al., 1989; Selvadurai et al., 1991).

The detection of IAA in OAB culture filtrates may depend on the growth phase of each isolate since IAA production has been shown to be directly proportional to the number of cells present in culture at the time of extraction (Tien et al., 1979). In addition, the use of TLC in this study was as a quantitative rather than qualitative measure of the production of IAA by the isolates. The absence of IAA on TLC may indicate sub-detectable levels which may be detectable using more sensitive procedures e.g. ELISA, mass spectrometry etc. The ecological significance of sub-detectable (by TLC) production of IAA by bacterial strains needs further investigation. It is likely that low levels of IAA produced/in situ within tissue are sufficient for the growth responses of the colonized tissue. Responses to high concentrations of applied IAA therefore are considered to be the result of relatively low incorporation of the compound into tissues rather than high requirements of the germinating orchid seedling.

IAA was also produced by isolates that had an inhibitory effect on the symbiotic germination of *P. vittata*. It is likely that these isolates also produce other growth regulators (such as gibberellins and cytokinins) or toxins in culture which may be deleterious to mycorrhizal synthesis. Gibberellins, cytokinins and IAA have often been detected simultaneously in the culture fractions of many bacterial genera (Barea et al., 1976; Cacciari et al., 1989; Tien et al., 1979). Similarly, soil bacteria are also known to be capable of producing substances toxic to plant growth (Alstrom and Gerhardson, 1989; Fredrickson et al., 1987).

Some substances of bacterial origin which are beneficial or inhibitory to orchid seed germination may have the opposite effect on the post-germination growth of the seedling. Mycorrhizal synthesis in asymbiotically germinated *Dactylorhiza incarnata* (L.) Soo seeds resulted in an increase in the concentration of cytokinins and IAA compared to non-mycorrhizal protocorms (Beyrle et al., 1991). In this case it was suggested that these substances were produced by the mycorrhizal fungus. This suggests that an exogenous supply of cytokinin may inhibit germination but benefit post-germination growth and development of an orchid. Since OAB were introduced at very low inoculum levels ($\sim 10^2$ to 10^3 cfu/plate), the biomass required for the production of effective concentrations of metabolites would have been possible only if there was rapid multiplication of the bacteria following inoculation. The response to bacterial inoculation therefore may have resulted from the induction of endogenous hormone production in the developing protocorm by the bacteria and/or the mycorrhizal fungus.

References

Alstrom S and Gerhardson B 1989 Plant and Soil 117, 157–165.

Arditti J and Ernst R 1984 *In* Orchid Biology – Reviews and Perspectives, III. Ed. J Arditti. pp 177–222. Ithaca Press, New York.

Arditti J et al. 1990. Lindl. 5, 249–255.

Barea J M et al. 1976 J. Appl. Bacteriol. 40, 129–134.

Barroso J et al. 1986 New Phytol. 103, 745–749.

Bentley J A 1962 Meth. Biochem. Anal. 9, 75–125.

Beyrle H et al. 1991 New Phytol. 117, 665–672.

Cacciari I et al. 1989 Plant and Soil 115, 151–153.

Fredrickson J K et al. 1987 Soil Biol. Biochem. 19, 127–134.

Healy M J R 1988 GLIM: An introduction. Clarendon Press, Oxford.

Hewlett-Packard 1987 Hp 5898A Microbial Identification System – Operation Manual. Hewlett-Packard Company, Route 41, Avondale, PA 19311, USA.

Ramsay R R et al. 1986 Lindl. 1, 203–214.

Sasser M 1990 *In* Methods in Phytobacteriology. Eds. Z Klement et al. pp 199–204. Akademiai Kiado, Budapest.

Selvadurai E L et al. 1991 Soil Biol. Biochem. 23, 401–403.

Tien T M et al. 1979 Appl. Env. Microbiol. 37, 1016–1026.

Wilkinson K G et al. 1989 New Phytol. 112, 429–435.

Wilkinson K G et al. 1990 Proc 4th Int. Mycol. Cong., Regensburg, Germany, Aug. 1990.

Wilkinson K G et al. 1993 Soil Biol. Biochem. (*In press*).

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