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ORIGINAL PAPER

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Promotion of plant growth by an auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots

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Abstract A plant-growth-promoting isolate of the yeast *Williopsis saturnus* endophytic in maize roots was found to be capable of producing indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA) in vitro in a chemically defined medium. It was selected from among 24 endophytic yeasts isolated from surface-disinfested maize roots and evaluated for their potential to produce IAA and to promote maize growth under gnotobiotic and glasshouse conditions. The addition of L-tryptophan (L-TRP), as a precursor for auxins, to the medium inoculated with *W. saturnus* enhanced the production of IAA and IPYA severalfold compared to an L-TRP-non-amended medium. The introduction of *W. saturnus* to maize seedlings by the pruned-root dip method significantly ($P<0.05$) enhanced the growth of maize plants grown under gnotobiotic and glasshouse conditions in a soil amended with or without L-TRP. This was evident from the increases in the dry weights and lengths of roots and shoots and also in the significant ($P<0.05$) increases in the levels of in planta IAA and IPYA compared with control plants grown in L-TRP-amended or non-amended soil. The plant growth promotion by *W. saturnus* was most pronounced in the presence of L-TRP as soil amendment compared to seedlings inoculated with *W. saturnus* and grown in soil not amended with L-TRP. In the glasshouse test, *W. saturnus* was recovered from inside the root at all samplings, up to 8 weeks after inoculation, indicating that the roots of healthy maize may be a habitat for the endophytic yeast. An endophytic isolate of *Rhodotorula glutinis* that was incapable of producing detectable levels of IAA or IPYA in vitro failed to increase the endogenous levels of IAA and IPYA and failed to promote plant growth compared to *W. saturnus*, although colonization of maize root tissues by *R. glutinis* was similar to that of *W. saturnus*. Both endophytic yeasts, *W. saturnus* and *R. glutinis*, were incapable of producing in vitro detectable levels of gibberellic acid, isopentenyl adenine, isopentenyl adenoside or zeatin in their culture filtrates. This study is the first published report to demonstrate the potential of an endophytic yeast to promote plant growth. This is also the first report of the production of auxins by yeasts endophytic in plant roots.

Keywords Biological fertilizers · Endophytes · Plant growth promotion · Plant growth regulators · Yeasts

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Introduction

Endophytic microorganisms have been defined as those that reside at some phases of their life cycle within living plant tissues without causing apparent damage to them (Petrini 1991) or which can be extracted from inner plant parts or isolated from surface-disinfested plant tissues (Hallmann et al. 1997). The role of endophytic microbial community in endophyte–plant associations has been intensively discussed (Hallmann et al. 1997; Stone et al. 2000; Sturz et al. 2000).

Endophytic bacteria and filamentous fungi have been isolated from surface-sterilized seeds, roots, stems, leaves, needles, twigs and barks of various symptomless plant species (Stone et al. 2000; Sturz et al. 2000). Microbial endophytes include both commensal microorganisms that have no direct effect on plants and beneficial microorganisms that could be used in the biological control of plant

pathogens or for plant growth promotion (Hallmann et al. 1997; Stone et al. 2000; Sturz et al. 2000).

The role of endophytic microorganisms in the promotion of plant growth has received increasing attention, and there is at present great interest in the introduction and/or manipulation of endophytic microorganisms to provide a consistent and effective increase in the productivity of crops (Sturz et al. 2000). Although growth promotion by endophytic bacteria (Sturz et al. 2000; Bacon and Hinton 2002) and endophytic filamentous fungi (Sivasithamparam 1998; Varma et al. 1999; Mucciarelli et al. 2003) have been reported, there appears to be no record in the literature on the use of endophytic yeasts for plant growth promotion.

Saprophytic yeasts are a common component of the mycoflora of aerial plant surfaces, including bark (Buck et al. 1998), leaves (Andrews and Buck 2002) and fruit surfaces (Arras et al. 2002) as well as rhizosphere soils (Bab'eva and Belyanin 1966; El-Tarabily 2004). Rhizosphere yeast strains, however, belonging to several genera, chiefly, *Sporobolomyces roseus* (Perondi et al. 1996), *Rhodotorula* sp. (Abd El-Hafez and Shehata 2001), *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* (El-Tarabily 2004) have been reported to promote plant growth. In addition, a variety of yeast genera has been used extensively for the biological control of post-harvest diseases of fruits and vegetables (Arras et al. 2002) to prevent moulding of stored grains (Petersson et al. 1999), to control wood-inhabiting fungi (Payne and Bruce 2001) and to control foliar diseases such as powdery mildews (Urquhart and Punja 2002).

The production of plant growth regulators (PGRs) has been suggested to be one of the mechanisms by which plant-growth-promoting microorganisms stimulate plant growth. Auxins are a class of PGRs known to stimulate both rapid (e.g. increases in cell elongation) and long-term (e.g. cell division and differentiation) responses in plants (Cleland 1990). Diverse soil microorganisms including bacteria (Arshad and Frankenberger 1998; Khalid et al. 2004), filamentous fungi (Kaldorf and Ludwig-Muller 2000; Floch et al. 2003) and yeasts (El-Tarabily 2004) are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. L-Tryptophan (L-TRP) is considered as a physiological precursor of auxin biosynthesis in both higher plants and microorganisms (Arshad and Frankenberger 1998). Exogenous application of L-TRP substantially increased auxin production in vitro by various bacteria (Khalid et al. 2004) and filamentous fungi (Frankenberger and Poth 1987).

Worldwide (Bashan et al. 2004) and currently in the United Arab Emirates (UAE) (El-Tarabily et al. 2003; Nassar et al. 2003), there is considerable interest in the application of biological fertilizers to reduce the inputs of chemical fertilizers. The main objectives of the present investigation were to (1) select from among endophytic yeasts from maize roots those capable of producing auxins in the presence or absence of L-TRP and examine the abilities of these isolates to promote maize growth under gnotobiotic conditions, (2) evaluate the endophytic potential of the most promising auxin-producing isolate to colonize maize roots and (3) observe the response of maize plants to the inoculation with endophytic yeasts in a soil amended with or without L-TRP under controlled glasshouse conditions by evaluating plant growth and levels of endogenous auxins in roots and shoots.

Materials and methods

Isolation of endophytic yeasts from surface-disinfested maize roots

Field soil under a maize crop, *Zea mays* L. cv. Merit (Asgrow Vegetable Seeds, California, USA), was collected from a farm located at Al-Ain city, 140 km east of Abu-Dhabi, UAE. Free-draining pots (20 cm in diameter) were filled with 8 kg of air-dried sieved soil. Maize seeds were surface disinfested by momentarily exposing to 70% ethyl alcohol for 5 min followed by 1.05% solution of commercial bleach for 4 min. The seeds were then subjected to eight washings with sterile distilled water. A surfactant (Tween 20, 0.05 ml l^{-1}, Sigma Chemical Co., St. Louis, MO, USA) was used in all the disinfestation procedures using hypochlorite. Surface-disinfested seeds were sown in pots at a depth of 1 cm, and the pots were placed in an evaporative-cooled glasshouse and maintained at 25±2°C. Pots were watered to container capacity and were fertilized every 10 days with inorganic liquid fertilizer (Thrive, Arthur Yates & Co. Limited, Milperra, NSW, Australia) (NPK 27:5.5:9) at the manufacturer's recommended rate. The experiment was replicated ten times with four plants in each replicate. After 3 weeks, plants were uprooted and transferred to the laboratory in coolers for immediate processing. To isolate endophytic yeasts, the severed roots cut from stems were rinsed in running tap water for 1 h to remove soil particles and surface contaminants and the fresh root weight recorded before further processing. Roots were soaked in sterile phosphate-buffered saline solution (PBS) (pH 7.0) for 10 min to equilibrate osmotic pressure and to prevent passive diffusion of sterilizing agents into the roots (Rennie et al. 1982).

Roots were surface disinfested by first exposing them to propylene oxide vapour for 25 min (Sardi et al. 1992). They were then soaked in 70% ethyl alcohol for 4 min followed by immersion in 1.05% solution of commercial bleach and shaken by hand for 5 min. The surface-disinfested roots were then rinsed ten times (5 min each rinse) in sterile phosphate buffer (PB) (Hallmann et al. 1997). To confirm that the surface disinfestation process was successful and to verify that no biological contamination from the surface of the maize was transmitted into the root tissues during maceration, sterility checks were carried out for each sample to monitor the effectiveness of the disinfestation procedures. For these checks, root impressions were taken (Sturz et al. 1998) and 0.2 ml from the final rinse was plated out on petri plates of tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA), potato dextrose agar (Difco) and yeast–malt–peptone–dextrose agar (YMPDA) (Wickerham 1951) amended with 250 μg ml^{-1} chloramphenicol (Sigma). The absence of bacterial and fungal, including

yeast growth, after 6 days of incubation in the sterility checks was taken to confirm that sterility and yeasts that were isolated were considered to be endophytic.

Roots were macerated in 100 ml of PB using a sterile mortar and pestle under aseptic conditions and then shaken for 1 h using a wrist-action shaker. The slurry was filtered through sterile filter papers, and the filtrate was serially diluted (10^{-2}–10^{-5}) in PB (Hallmann et al. 1997). Aliquots (0.2 ml) were spread with a sterile glass rod over the surface of YMPDA amended with chloramphenicol for the enumeration of the total endophytic yeast populations. Plates were dried in a laminar flow cabinet for 10 min before incubation at 25°C in the dark for 7 days. Three plates per dilution were made for each root sample. Population densities were expressed as $\log_{10}$ colony forming units (CFU) g^{-1} fresh root weight (Hallmann et al. 1997). All yeast isolates were transferred onto YMPDA, and the isolates were re-streaked twice to ensure purity. The cells of all isolates from four plates were removed from the culture surface with a sterile spatula and stored in 20% glycerol (cryoprotectant) at −80°C (Wellington and Williams 1978).

In vitro screening for indole-3-acetic acid production

The aim of this experiment was to screen the obtained 24 endophytic yeast isolates for their ability to produce indole-3-acetic acid (IAA) in glucose peptone broth (GPB) (di Menna 1957) amended with or without L-TRP (Sigma). Erlenmeyer flasks (100 ml), each containing 20 ml of sterile GPB, were amended with 5 ml of 5% filter-sterilized L-TRP (Millipore membranes, pore size 0.22 μm, Millipore Corporation, Bedford, MA, USA) (Khalid et al. 2004). The flasks were inoculated with 2 ml of each of the isolated yeasts prepared from a 5-day-old shake GPB culture of approximately 1×10^{8} CFU ml^{-1}, covered with aluminium foil and incubated on a shaker (Model G76, New Brunswick Scientific, Edison, NJ, USA) at 250 rpm at 25°C in the dark for 7 days. Non-inoculated flasks served as controls. After incubation, the suspension from each flask was centrifuged for 30 min at 12,000×*g*. The supernatant was filtered through sterile Millipore membranes (pore size 0.22 μm) and collected in sterile tubes. The culture supernatants (3 ml) were pipetted into test tubes, and 2 ml of Salkowski reagent (2 ml of 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) were added to it (Gordon and Weber 1951). The tubes containing the mixture were left for 30 min for red colour development. The intensity of the colour was determined by optical density at 530 nm using a scanning spectrophotometer (UV-2101/3101 PC, Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). Similarly, colour was also developed in standard solutions of IAA, and a standard curve was established and auxin compounds were expressed as IAA equivalents (Gordon and Weber 1951). Four independent replicates of each isolate were analysed. For the high-performance liquid chromatography (HPLC) detection of auxins, including IAA and indole-3-pyruvic acid (IPYA), the parameters described by Tien et al. (1979) were employed. The isolates were grown in GPB and the details on the inoculation of the broth, extraction of auxins and HPLC analysis were described by Tien et al. (1979). Eight independent replicates were analysed.

Assessment of growth promotion under gnotobiotic conditions

All eight isolates that produced detectable levels of IAA (isolates 1, 4, 7, 11, 12, 15, 18 and 23) were further tested under gnotobiotic conditions to study the effectiveness of seedling inoculation with the endophytic yeast isolates in the presence or absence of L-TRP on maize root and shoot growth using sterilized sand as described by Chanway et al. (1991). Two non-IAA-producing endophytic yeast isolates (16 and 20) were also included for comparison. Sand was acid-washed overnight in 1:1 (w/v) sand–6 M HCl, rinsed with tap water for 30 min, rinsed ten rinses with deionised water for 40 min and autoclaved for 40 min. Glass tubes (300×35 mm in diameter) were filled with autoclaved, acid-washed sand (280 g), moistened with distilled water and the tubes autoclaved again for 40 min. Filter-sterilized (pore size 0.22 μm, Millipore) nutrient solution (65 ml) described by Arsac et al. (1990) amended with or without L-TRP (3 mg kg^{-1} sand) was added to each tube as a single application after planting the seedlings as recommended by Frankenberger et al. (1990). The choice of this optimum concentration was based on a preliminary study using various levels of L-TRP ranging from 3×10^{-4} to 30 mg kg^{-1} sand to determine their effects on maize growth (data not shown).

Seedlings were inoculated with the yeast suspensions using the pruned-root dip method as described by Musson et al. (1995). Healthy maize seeds were surface disinfested as described above and pre-germinated on moist-sterile filter paper at 25°C in the dark for 2 days to obtain uniform seedlings. When roots were about 15 mm long, the root tips (3 mm) were trimmed using a sterilized scalpel to facilitate the uptake of the inoculum (Musson et al. 1995; Bressan and Borges 2004). The seedlings were placed in sterile plastic cups at 25°C for 3 h with only their roots in contact with the inoculum suspension of each isolate at 10^{8} CFU ml^{-1}. As controls, seedlings with severed root tips were treated with autoclaved inoculum. The maize seedlings with or without the living yeast isolates were then planted into the tubes under aseptic conditions and watered as needed with sterile distilled water. The tubes were incubated in a growth room under a 16-h photoperiod, 180–200 μmol m^{-2} s^{-1} fluorescent light and a 25:20°C light–dark temperature cycle. Two weeks after transplantation, the plants were harvested, washed and separated into roots and shoots. The lengths and weights of shoots and roots were used to measure the effects of endophytic yeasts on plant growth. Each treatment was independently replicated eight times with one seedling in each replicate.

Identification of the selected yeast isolates

On the basis of the results obtained from in vitro IAA production and based upon the performance of the yeast isolates on the growth of maize seedlings under gnotobiotic conditions, only two isolates (isolates 4 and 16) were selected and tested in pot trials to observe their endophytic colonization in the roots and to study their effects on growth and development of maize plants under controlled glasshouse conditions. The outstanding IAA-producing isolate that provided the best growth-promotion activities under gnotobiotic conditions was selected and was identified as *Williopsis saturnus* (Klöcker) Zender (isolate 4). An endophytic non-IAA-producing isolate (isolate 16), which did not promote maize growth, was included in the present study for comparison and was identified as *R. glutinis* (Fresenius) Harrison. Identification based on morphological, cultural, physiological and biochemical characteristics was carried out by the Yeast Division, Centraalbureau voor Schimmelcultures, Delft, the Netherlands.

Quantitative determination of PGRs by HPLC

For the detection of gibberellic acid (GA_3) and the cytokinins [isopentenyl adenine (iPa), isopentenyl adenoside (IPA) and zeatin (Z)] in the individual extracts of *W. saturnus* and *R. glutinis*, the parameters described by Tien et al. (1979) were used. The isolates were grown in GPB and the details on the inoculation of the broth, extraction of PGRs and HPLC analysis were described by Tien et al. (1979). Eight independent replicates of each isolate were analysed.

Glasshouse trials

Inoculum production

W. saturnus and *R. glutinis* were grown in 250-ml Erlenmeyer flasks containing 100 ml of sterilized yeast–malt–peptone–dextrose broth (YMPDB) on a shaker (New Brunswick Scientific) at 200 rpm at 25°C in the dark for 5 days. Yeast cells were harvested by centrifugation (12,000×*g* at 20°C for 20 min), and the pellet was suspended in 10 ml of sterile PB. A dilution series was made of each suspension in PB, and 0.2 ml each of the 10^{-6}, 10^{-7} and 10^{-8} dilutions was spread onto YMPDA. Plates were incubated at 25°C in the dark for 5 days before determining the CFU ml^{-1}. A concentration of approximately 10^8 CFU ml^{-1} of each isolate was used as inoculum.

Estimation of internal root colonization

Pot trials were designed to assess the internal colonization of maize roots by *W. saturnus* and *R. glutinis* after seedling inoculation through the pruned-root dip method described above. Cycloheximide-resistant mutants of *W. saturnus* and *R. glutinis* were prepared using the method described by Suslow and Schroth (1982). The obtained mutants were compared with their wild types in relation to their ability to produce auxins. None of these mutants differed morphologically from their parental strains, and all mutants had identical growth rates and auxin-production ability with their parental strains. Maize seedlings prepared as described above and inoculated with *W. saturnus* or *R. glutinis* were then planted into free-draining pots (30 cm in diameter) filled with 18 kg of sieved soil collected from the same maize field described above. The pots were placed in an evaporative-cooled glasshouse, maintained at 25± 2°C, watered to container capacity and fertilized with inorganic liquid fertilizer as described above. Every week after planting (1–8), roots were sampled from the soil, washed thoroughly in tap water, surface disinfested as described above and the population densities of *W. saturnus* or *R. glutinis* (log_{10} CFU g^{-1} fresh root weight) were determined using YMPDA amended with cycloheximide (50 μg ml^{-1}, Sigma). Each treatment was replicated five times with two plants in each replicate for each sampling.

Glasshouse in vivo trials

The effect of *W. saturnus* and *R. glutinis* on maize growth was further tested in vivo in soil amended with or without L-TRP. Soil was collected from the same maize field and sieved as described above. The chemical characteristics of the soil were analysed as described previously in El-Tarabily et al. (1996). The soil characteristics were pH 7.2 (in 0.01 M $CaCl_2$), electrical conductivity 1.04 dS m^{-1} and organic C 0.81%; the following nutrients are expressed in milligrams per kilogram soil: bicarbonate extractable K^+ and P 285 and 76, respectively; NO_3^-–N 16, NH_4^+–N 45; SO_4^{2-} 76 and Fe 432. Free-draining pots (30 cm in diameter) were filled with 18 kg of soil. Control seedlings and seedlings inoculated with *W. saturnus* or *R. glutinis* prepared as described above by the pruned-root dip method were planted. In the treatments, which included the amendment of 3.0 mg L-TRP kg^{-1} soil, the chemical was applied in solution to soil as a single application as described above. In general, there was a total of eight treatments as follows: (1) seedlings inoculated with autoclaved *W. saturnus* in L-TRP-non-amended soil, (2) seedlings inoculated with autoclaved *W. saturnus* in L-TRP-amended soil, (3) seedlings inoculated with autoclaved *R. glutinis* in L-TRP-non-amended soil, (4) seedlings inoculated with autoclaved *R. glutinis* in L-TRP-amended soil, (5) seedlings inoculated with *W. saturnus* in L-TRP-non-amended soil, (6) seedlings inoculated with *W. saturnus* in L-TRP-amended soil, (7) seedlings inoculated with *R. glutinis* in L-TRP-non-amended soil and (8) seedlings inoculated with *R. glutinis* in L-TRP-amended soil. Each treatment was replicated 12 times with two plants per replicate. The pots were placed in an evaporative-cooled glasshouse and maintained at 25°C± 2°C for 8 weeks. The pots were watered to container capacity and fertilized with the inorganic fertilizer as described

above. Plant growth was monitored by recording the dry weights and lengths of roots and shoots at the time of harvest.

Extraction and HPLC analysis of endogenous auxins in maize roots and shoots

The extraction of endogenous auxins (IAA and IPYA) were carried out from tissues of the terminal part of the root and shoot systems using the method described by Guinn et al. (1986). Briefly, the tissues were quickly frozen at −85°C and ground in cold 80% extracting solvent (methanol–butylated hydroxytoluene–sodium ascorbate). The macerate was then transferred to a flask with fresh extracting solvent, and the volume was adjusted to 20 ml and filtered. The filtrate was evaporated to the aqueous phase in a rotary flash evaporator at 35°C. The aqueous phase was then adjusted to pH 8 with K_2HPO_4, the sample was partitioned three times with equal volumes of washed ethyl acetate–butylated hydroxytoluene and the aqueous phase was adjusted to pH 2.8 with H_3PO_4 (Sigma). The acidified solution was passed through C_{18} Sep-Pak cartridge (Waters Corporation, Milford, MA, USA) to trap auxins. Auxins were then eluted with NH_4OH (Sigma) and the pH quickly adjusted to 2.8 with H_3PO_4. The aqueous eluted phase was partitioned three times, each with a 10-ml portion of washed diethyl ether–butylated hydroxytoluene. The ether was evaporated by rotary flash evaporation at 40°C, the residue was immediately dissolved in methanol and the sample was injected into the HPLC for the determination of the endogenous auxins using the parameters described by Tien et al. (1979). HPLC chromatograms were produced by injecting 10 μl of the methanol-dissolved sample onto a 10-μm reverse-phase column (Waters Associates, Bondapak C_{18}, 4 mm×30 cm) in a Waters Associates liquid chromatography system equipped with a differential ultraviolet detector operated at 280 nm. Two isocratic solvent systems were used to separate auxins as described by Tien et al. (1979). The concentrations of auxins were obtained by comparing their respective peak areas in the unknown sample with their corresponding areas obtained with the authentic samples (Sigma) of a known concentration. Eight independent replicate samples were analysed.

Light and transmission electron microscopy

Two weeks after inoculation with *W. saturnus*, the maize roots were washed with sterile distilled water, fixed in 2% glutaraldehyde in 0.17 M PB (pH 7.2) at room temperature under vacuum for 24 h and were subjected to four cycles of washing in the same buffer. Samples were postfixed in 1% osmium tetroxide in 0.17 M PB for 2 h, rinsed three times with distilled water, dehydrated through a series of ethanol solutions, embedded in epoxy resin (Epon 812, Agar Scientific, UK) and polymerised at 60°C for 24 h (Millonig 1976).

Semithin transverse sections (0.5 μm) were cut with glass knives on an ultramicrotome (Leica Ultracut, Sweden) and stained with 0.1% toluidine blue for observation under a light microscope. Sections were examined using an Olympus BH-2 microscope (Olympus Optical Co., Ltd, Tokyo, Japan). For transmission electron microscopy, ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate and examined with a Philips CM10 transmission electron microscope (the Netherlands) operating at 80 kV.

Statistical analysis

All experiments were arranged in a completely randomized block design. Population data were transformed into $\log_{10}$ CFU g^{-1} fresh root weight. Data were subjected to analysis of variance (ANOVA), and treatment means were compared using Fisher's Protected LSD Test at P=0.05. Superanova (Abacus Concepts Inc., Berkeley, CA, USA) was used for all analyses.

Results

Isolation of endophytic yeasts and in vitro screening for IAA production

The populations of endophytic yeasts in maize roots were $\log_{10}$ 3.43 (±SE 0.12) CFU g^{-1} fresh root weight. No contamination was found in the sterility checks, indicating that the surface disinfestation procedures were adequate. A total of 24 yeast isolates were obtained from the maize root triturate. Auxins (expressed as IAA equivalents in micrograms per milliliter using colorimetric analysis) were detected only in liquid cultures of eight yeasts out of the 24 isolates (Table 1). Different yeast isolates varied greatly in their efficiency for IAA production in GPB medium both in the presence or absence of L-TRP (Table 1). In the presence of L-TRP, yeast efficiency for IAA production was enhanced by severalfold (Table 1). Two of the isolates (9 and 21) that did not produce detectable levels of IAA in L-TRP-non-amended medium produced a small amount of IAA when the medium was amended with L-TRP (Table 1). The remaining isolates were found to be non-IAA-producing and effected no colour change after the addition of the reagent to their culture filtrates in the presence or absence of L-TRP and were easily distinguished from IAA-producing isolates, which formed a dark red colour.

Assessment of growth promotion under gnotobiotic conditions

Maize seedling vigour as measured by root and/or shoot lengths and weights in L-TRP-amended or non-amended soil was significantly (P<0.05) increased by the inoculation with IAA-producing yeast isolates compared to that of the

Table 1 In vitro indole-3-acetic acid (IAA) production by endophytic yeast isolates in glucose-peptone broth (GPB) amended with or without L-tryptophan (L-TRP) after 7 days of incubation at 25°C

Isolate number	IAA equivalents (μg ml^{-1})	
	Without L-TRP	With L-TRP
Yeast 1	2.84 b A	18.71 h B
Yeast 4 (*W. saturnus*)	9.67 h A	22.51 j B
Yeast 7	4.31 c A	15.40 e B
Yeast 9	0.00 a A	3.45 a B
Yeast 11	7.50 g A	17.62 g B
Yeast 12	6.51 f A	13.34 d B
Yeast 15	4.82 d A	9.79 c B
Yeast 18	7.63 g A	19.72 i B
Yeast 21	0.00 a A	4.70 b B
Yeast 23	5.85 e A	16.32 f B

Values are means of four replicates, and the values with the same lower or same upper case letter within a column or a row, respectively, are not significantly (P>0.05) different according to Fisher's Protected LSD Test

control or seedlings inoculated with non-IAA-producing yeast isolates (16 and 20) (Table 2). Results also showed that the addition of L-TRP supported significantly (P< 0.05) better root and shoot development than L-TRP-non-amended soil in the presence of yeast isolates (Table 2). Different isolates of endophytic yeasts had variable effects on root and shoot growth (Table 2). Of the eight IAA-producing isolates, the most promising growth-promoting isolate (*W. saturnus*) that provided the best growth promotion in the presence or absence of L-TRP (Table 2) was chosen for further glasshouse studies. *R. glutinis* was included in this study as endophytic non-IAA-producing isolate for comparison with *W. saturnus*.

Production of PGRs by *W. saturnus* and *R. glutinis*

Auxins production by *W. saturnus* was also confirmed by HPLC analysis. The study revealed that *W. saturnus*, which produced the highest level of IAA in the colorimetric analysis, produced IAA (=13.25 and 28.62 μg ml^{-1}) (SE=0.32 and 0.63) and IPYA (=5.41 and 7.53 μg ml^{-1}) (SE=0.15 and 0.26) using HPLC analysis in medium amended with or without L-TRP, respectively. Neither IAA nor IPYA were detected with either *R. glutinis* or the sterile broth (control). The culture extracts of the *W. saturnus* or *R. glutinis* isolates did not show the presence of GA_3, iPa, IPA or Z.

Estimation of internal root colonization

The cycloheximide-resistant mutants of *W. saturnus* and *R. glutinis* were isolated from the surface-disinfested maize roots, indicating that these strains were endophytic. *W. saturnus* and *R. glutinis* maintained their endophytic colonizing abilities and were isolated from healthy maize roots at all samplings until week 8 (Table 3). An initial increase in colonies of *W. saturnus* after 1, 2 and 3 weeks was followed by a decrease at the fourth week (Table 3). However, at and after week 5 and up to week 8, its populations increased again (Table 3). For *R. glutinis*, an initial increase in its populations after 1 and 2 weeks was followed by a decrease after week 3 (Table 3). However, from week 4 and up to week 8, there was an increase in the population of *R. glutinis* within the roots (Table 3).

Glasshouse in vivo trials

Plants inoculated with *W. saturnus* in L-TRP-amended soil (treatment 6) or *W. saturnus* in L-TRP-non-amended soil

Table 2 Effect of indole-3-acetic acid (IAA)-producing endophytic yeast isolates on maize growth under gnotobiotic conditions

Isolate number	Root length (cm)		Shoot length (cm)		Root dry weight (g)		Shoot dry weight (g)	
	−L-TRP	+L-TRP	−L-TRP	+L-TRP	−L-TRP	+L-TRP	−L-TRP	+L-TRP
Control	9.57 a A	12.97 a B	7.71 a A	9.23 a B	0.059 a A	0.079 a B	0.105 a A	0.127 a B
Yeast 1	15.32 bc A	18.21 bcd B	11.32 bc A	14.58 bc B	0.097 b A	0.131 b B	0.163 b A	0.208 b B
Yeast 4 (*W. saturnus*)	17.50 d A	21.64 e B	14.08 e A	17.53 f B	0.146 g A	0.198 g B	0.226 e A	0.289 g B
Yeast 7	15.16 bc A	19.13 d B	11.93 bcd A	15.36 de B	0.098 bc A	0.145 c B	0.177 bc A	0.210 bc B
Yeast 11	14.65 b A	18.41 bcd B	12.31 d A	15.61 e B	0.107 cde A	0.156 de B	0.167 b A	0.217 cd B
Yeast 12	14.68 b A	18.13 bcd B	12.12 cd A	15.45 de B	0.115 ef A	0.162 ef B	0.174 bc A	0.221 de B
Yeast 15	14.45 b A	17.50 b B	11.15 b A	14.76 bcd B	0.106 bcd A	0.149 cd B	0.182 cd A	0.213 bc B
Yeast 18	15.71 c A	17.90 bc B	11.56 bcd A	14.38 b B	0.109 def A	0.157 e B	0.184 cd A	0.225 e B
Yeast 23	14.93 bc A	18.83 cd B	11.68 bcd A	15.13 cde B	0.118 f A	0.167 f B	0.193 d A	0.241 f B
Yeast 16 (*R. glutinis*)	10.05 a A	12.35 a B	7.35 a A	9.36 a B	0.054 a A	0.075 a B	0.098 a A	0.129 a B
Yeast 20	9.80 a A	12.61 a B	7.13 a A	9.68 a B	0.056 a A	0.077 a B	0.102 a A	0.132 a B

Inoculated maize seedlings were grown in glass tubes containing sterilized sand with or without L-tryptophan (L-TRP) for 2 weeks. Yeasts 16 and 20 are non-IAA-producing isolates. Values are means of eight replicates, and the values with the same lower or same upper case letter for each growth measurement within a column or a row, respectively, are not significantly (P>0.05) different according to Fisher's Protected LSD Test

Table 3 Total populations of the endophytic yeasts *W. saturnus* and *R. glutinis* at different times of sampling of roots of maize grown under glasshouse conditions

Time after inoculation (weeks)	Total population (log_{10} CFU g^{-1} fresh root weight)	
	(*W. saturnus*)	(*R. glutinis*)
1	2.48 a A	2.36 a A
2	2.92 b A	2.84 b A
3	3.48 c A	2.58 ab B
4	3.11 bc A	3.30 c A
5	4.10 d A	3.70 d A
6	4.67 e A	4.13 e A
7	5.16 f A	4.63 f A
8	5.85 g A	5.12 g B

Values are means of five replicates for each sampling, and the values with the same lower or same upper case letter within a column or a row, respectively, are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test

(treatment 5) significantly ($P<0.05$) enhanced the growth of maize plants as evidenced by the increase in the dry weights and lengths of roots and shoots compared with control plants grown in L-TRP-non-amended soil or L-TRP-amended soil (treatments 1 and 2, respectively) (Table 4). Responses to treatment 6 (seedlings inoculated with *W. saturnus* in L-TRP-amended soil) were the best among the treatments attempted. In this treatment, there were significant ($P<0.05$) increases in lengths and dry weights of roots and shoots compared to the treatments that received the application of autoclaved *W. saturnus* (treatment 2) or autoclaved *R. glutinis* (treatment 4) in L-TRP-amended soil or autoclaved *W. saturnus* (treatment 1) or autoclaved *R. glutinis* (treatment 3) in L-TRP-non-amended soil (Table 4). There was a significant ($P<0.05$) increase in the root and shoot growth characteristics of maize plants inoculated with autoclaved inoculum (control) (treatments 2 and 4) grown in L-TRP-amended soil compared to the treatments that received the application of autoclaved inoculum (control) (treatments 1 and 3) grown in L-TRP-non-amended soil (Table 4).

The application of non-IAA-producing isolate of *R. glutinis* in L-TRP-amended soil (treatment 8) significantly ($P<0.05$) enhanced the growth and development of maize plants compared to the treatments that received the application of *R. glutinis* in L-TRP-non-amended soil (treatment 7) (Table 4). However, these treatments performed less significantly ($P<0.05$) than the treatments that received the application of IAA-producing isolate of *W. saturnus* in L-TRP-amended soil (treatment 6) or in L-TRP-non-amended soil (treatment 5) (Table 4).

There were no significant ($P>0.05$) differences between the root or shoot growth characteristics of maize plants inoculated with *R. glutinis* in L-TRP-amended soil (treatment 8) and its control (treatment 4) or between maize plants inoculated with *R. glutinis* in L-TRP-non-amended soil (treatment 7) and its control (treatment 3) (Table 4).

Measurement of endogenous auxins from roots and shoots

Plants inoculated with *W. saturnus* and grown in L-TRP-amended soil (treatment 6) had significantly ($P<0.05$) higher levels of endogenous IAA and IPYA than those plants inoculated with *W. saturnus* and grown in L-TRP-non-amended soil (treatment 5) or the control plants grown in L-TRP-non-amended soil or L-TRP-amended soil in both roots and shoots (treatments 1 and 2), respectively (Table 5). There was a significant ($P<0.05$) increase in the endogenous IAA and IPYA contents of maize plants inoculated with autoclaved inoculum (control) (treatments 2 and 4) grown in L-TRP-amended soil compared to the treatments which received the application of autoclaved inoculum (control) (treatments 1 and 3) grown in L-TRP-non-amended soil (Table 4).

Plants inoculated with *R. glutinis* and grown in L-TRP-amended soil (treatment 8) had significantly ($P<0.05$) higher endogenous IAA or IPYA compared to the treatment that received the application of *R. glutinis* and were grown in L-TRP-non-amended soil (treatment 7) (Table 5). On the other hand, plants inoculated with *R. glutinis* and grown in

Table 4 Effect of application of endophytic yeasts *W. saturnus* and *R. glutinis* on growth characteristics of maize grown in an evaporative-cooled glasshouse maintained at 25±2°C

Treatment	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)
(1) Autoclaved *W. saturnus* in L-TRP-non-amended soil	72.31 a	116.13 a	12.37 a	26.19 a
(2) Autoclaved *W. saturnus* in L-TRP-amended soil	83.41 b	131.81 b	17.80 b	34.35 b
(3) Autoclaved *R. glutinis* in L-TRP-non-amended soil	70.03 a	117.42 a	13.89 a	25.44 a
(4) Autoclaved *R. glutinis* in L-TRP-amended soil	80.76 b	133.59 b	18.50 b	35.74 b
(5) *W. saturnus* in L-TRP-non-amended soil	94.04 c	152.21 c	22.61 c	42.29 c
(6) *W. saturnus* in L-TRP-amended soil	103.17 d	163.71 d	25.69 d	47.53 d
(7) *R. glutinis* in L-TRP-non-amended soil	68.69 a	119.51 a	12.91 a	27.32 a
(8) *R. glutinis* in L-TRP-amended soil	82.71 b	134.28 b	16.87 b	36.19 b

Inoculated maize seedlings were grown in soil amended with or without L-tryptophan (L-TRP) for 8 weeks. Values are means of 12 replicates, and the values with the same letter within each column are not significantly ($P>0.05$) different according to Fisher's Protected LSD Test

Table 5 Effect of application of endophytic yeasts *W. saturnus* and *R. glutinis* on the levels of endogenous indole-3-acetic acid (IAA) (μg 100 g^{-1} dry weight) and indole-3-pyruvic acid (IPYA) (μg 100 g^{-1} dry weight) in the roots and shoots of maize plants grown in an evaporative-cooled glasshouse maintained at 25±2°C

Treatment	Roots		Shoots	
	IAA	IPYA	IAA	IPYA
(1) Autoclaved *W. saturnus* in L-TRP-non-amended soil	12.87 a	1.68 a	28.51 a	5.41 a
(2) Autoclaved *W. saturnus* in L-TRP-amended soil	22.51 b	3.83 b	47.12 b	8.52 b
(3) Autoclaved *R. glutinis* in L-TRP-non-amended soil	11.03 a	1.71 a	28.13 a	5.38 a
(4) Autoclaved *R. glutinis* in L-TRP-amended soil	23.98 b	3.74 b	45.51 b	8.06 b
(5) *W. saturnus* in L-TRP-non-amended soil	50.78 c	5.57 c	73.62 c	16.11 c
(6) *W. saturnus* in L-TRP-amended soil	78.45 d	7.71 d	115.37 d	23.72 d
(7) *R. glutinis* in L-TRP-non-amended soil	14.35 a	1.55 a	25.24 a	5.12 a
(8) *R. glutinis* in L-TRP-amended soil	23.67 b	3.59 b	42.11 b	8.11 b

Inoculated maize seedlings were grown in soil amended with or without L-tryptophan (L-TRP) for 8 weeks. Values are means of eight replicates, and the values with the same letter within each column are not significantly (P>0.05) different according to Fisher's Protected LSD Test

L-TRP-amended soil (treatment 8) had significantly (P< 0.05) lower endogenous IAA or IPYA compared to the treatment that received the application of *W. saturnus* and was grown in L-TRP-amended soil (treatment 6) (Table 5). There were no significant (P>0.05) differences between the endogenous IAA and IPYA contents of roots or shoots of maize plants inoculated with *R. glutinis* in L-TRP-amended soil (treatment 8) compared to its control (treatment 4) or between maize plants inoculated with *R. glutinis* in L-TRP-non-amended soil (treatment 7) and its control (treatment 3) (Table 4). Overall, the levels of IAA and IPYA observed in the shoots were relatively higher than those in the roots in all treatments (Table 5).

Light and transmission electron microscopy

Microscopy of the inoculated maize roots showed the presence of yeast cells of *W. saturnus* in the intercellular spaces of the cortex and within the cells of the cortex and

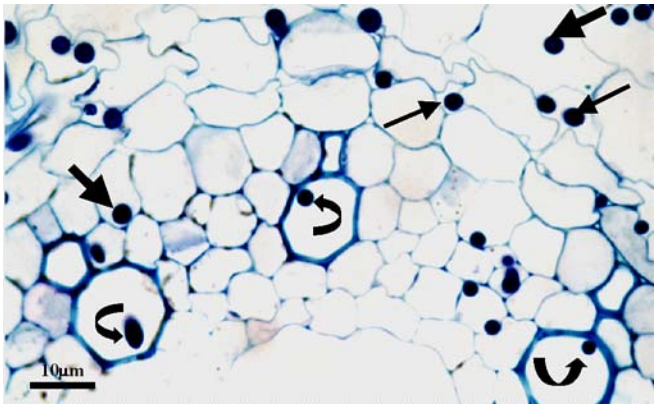

Fig. 1 Light micrograph of semithin sections of 2-week-old maize root inoculated with *W. saturnus* and stained with 0.1% toluidine blue showing the distribution of yeast cells within the root cortex (*thick arrows*), intercellular spaces (*thin arrows*) and xylem vessels (*curved arrows*) (×1000, scale bar=10 μm)

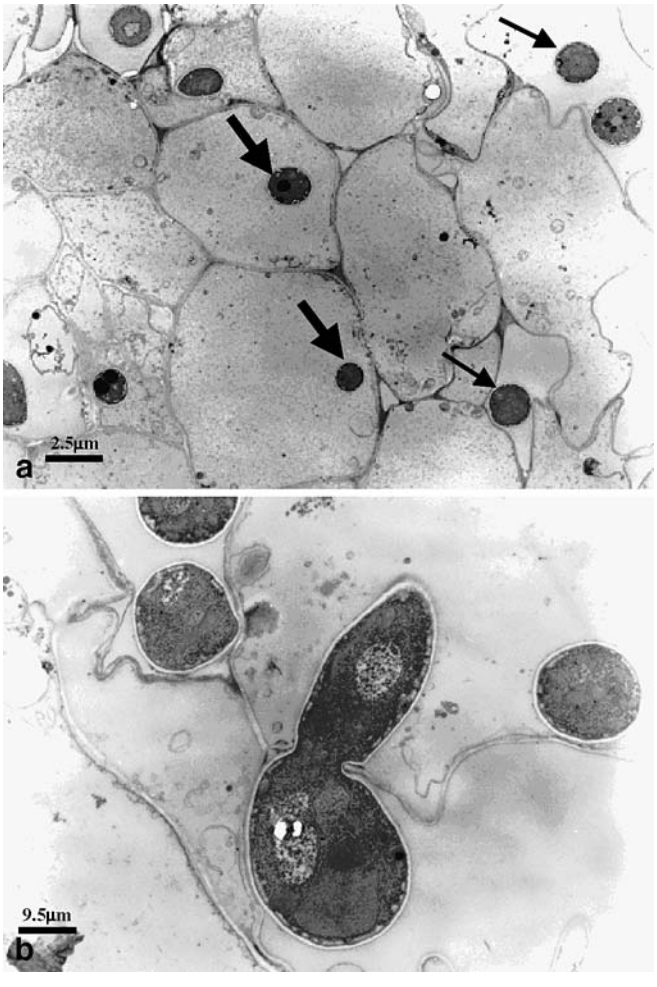

Fig. 2 Transmission electron micrograph of ultra-thin sections of 2-week-old maize root inoculated with *W. saturnus* showing **a** the distribution of yeast cells within the root cortex (*thick arrows*) and intercellular spaces (*thin arrows*) (×4180, scale bar=2.5 μm) and **b** penetration of a neighbouring root cortical cell by a budding yeast cell (×12559, scale bar=9.5 μm)

pith as well as in the xylem vessels (Figs. 1 and 2a). Some of the yeast cells were found to be in the process of budding (Fig. 2b), whereas certain others produced pseudo-mycelium.

Discussion

In the present study, an endophytic isolate of the yeast *W. saturnus* capable of producing relatively high levels of IAA and IPYA in the growth medium amended with L-TRP significantly promoted growth of maize under gnotobiotic and glasshouse conditions in the presence or absence of L-TRP in comparison to the other endophytic yeasts tested. The combined application of *W. saturnus* and L-TRP was more effective in improving maize growth as compared with that where each was applied alone. In our study, *W. saturnus* was selected from among 24 endophytic yeast isolates based upon in vitro IAA production and growth-promoting activity under gnotobiotic conditions. This study is the first record of plant growth promotion by auxin-producing yeasts endophytic in plant roots.

The exogenous application of L-TRP combined with the IAA-producing isolate of *W. saturnus* enhanced plant growth best compared to non-amended soil. This superiority could also be explained by the additional input of auxins by the indigenous soil microflora following the L-TRP amendment of the soil, in addition to the ability of roots to absorb auxins produced in the soil and the ability of the plant to absorb L-TRP and convert it with its own enzymes to auxins. Zahir et al. (1997) investigated the effectiveness of precursor–inoculum interactions. They studied the effect of inoculation with auxin-producing *Azotobacter*, both in the presence and absence of L-TRP, on potato yield and reported that the combined application of *Azotobacter* and L-TRP amendment was more effective than their application alone in increasing the tuber and straw yield of potato. This is in agreement with our results.

Although a variety of yeast genera have been reported from maize rhizosphere (Bab'eva and Belyanin 1966; Gomes et al. 2003), only a few attempts have been made to use rhizosphere yeasts as biological fertilizers. The application of *S. roseus* in Brazil (Perondi et al. 1996), *Rhodotorula* sp. (Abd El-Hafez and Shehata 2001), *C. valida*, *R. glutinis* and *T. asahii* in Egypt (El-Tarabily 2004) has been reported to promote wheat, tomato and sugar beet growth, respectively. None of these studies, however, tested endophytic yeasts for plant growth promotion. In the present study, a third of our isolates was capable of producing IAA from L-TRP-amended medium. The only report of endophytic IAA-producing yeast dealt only with *Pichia spartinae* (Nakamura et al. 1991) and gave no indication on the proportion of the isolates capable of producing IAA.

Endophytic bacteria (Bacon and Hinton 2002; Zinniel et al. 2002) and filamentous fungi (Fisher et al. 1992) have been previously isolated from maize roots; however, the present study is the first record to isolate endophytic yeasts from maize roots. Although unidentified endophytic yeasts from banana roots (Cao et al. 2002), *Acrostichum aureum* rhizomes (Maria and Sridhar 2003), rice leaves (Tian et al. 2004), tomato leaves (*Rhodotorula* sp.) (Larran et al. 2001), wheat leaves (*Rhodotorula rubra* and *Cryptococcus* sp.) (Larran et al. 2002) and from within the culm vascular spaces of cordgrass *Spartina alteniflora* (*P. spartinae*) (Nakamura et al. 1991) have been isolated, they were not tested for their potential as plant growth promoters.

In the present study, although the growth of both roots and shoots were promoted by *W. saturnus*, there were no significant differences between the growth responses of the root and shoot of maize plants inoculated with *R. glutinis* in L-TRP-amended soil and its control or between maize plants inoculated with *R. glutinis* in L-TRP-non-amended soil and its control. However, the treatment where *R. glutinis*-inoculated seedlings were grown in L-TRP-amended soil showed a significant growth promotion compared to *R. glutinis*-inoculated seedlings grown in L-TRP-non-amended soil. This is considered to have been the result of the utilization by indigenous soil microorganisms of the L-TRP applied. This emphasised the importance of the presence of a precursor in the soil environment required for significant plant growth promotion.

Although the non-IAA-producing isolate of *R. glutinis* was an endophyte of maize roots, it was unable to promote maize growth under gnotobiotic or glasshouse conditions in the presence or absence of L-TRP compared to *W. saturnus*. This indicates that the success of *W. saturnus* may be related not only to its ability to synthesize IAA from L-TRP but also through the activation of endogenous auxins in planta. It is noteworthy that both *W. saturnus* and *R. glutinis* were incapable of producing detectable levels of GA_3, iPa, IPA or Z in vitro. This further supports the probability that the promotion effects observed in the present study by *W. saturnus* are mainly due to the activity of auxins. Plant growth promotion by other microorganisms has been recorded wherein the effective isolates were capable of producing only auxins (Barea et al. 1976; Strzelczyk and Pokojska-Burdziej 1984). It is, however, possible that growth-promoting factors other than those tested may also have had a role in the growth promotion of the plant observed.

The observed increase in plant growth of maize by an endophytic yeast is supported by other observations in which endophytic bacteria (Bastian et al. 1998; Bacon and Hinton 2002) and filamentous fungi (Sivasithamparam 1998; Varma et al. 1999; Floch et al. 2003; Mucciarelli et al. 2003) were also shown to enhance plant growth. *W. saturnus* used in the present study was shown to produce IAA and IPYA that affect plant growth. Several endophytic bacteria (Bashan and Holguin 1997; Bastian et al. 1998), filamentous fungi (Lu et al. 2000) and yeasts (Nakamura et al. 1991) have been reported to produce auxins in vitro. Auxins produced by rhizosphere bacteria (Bashan et al. 2004; Khalid et al. 2004), filamentous fungi (Frankenberger and Poth 1987) and yeasts (El-Tarabily 2004) have been reported to enhance growth and improve yields of host plants.

The addition of exogenous L-TRP is known to substantially increase the in vitro auxin production by bacteria

(Arshad and Frankenberger 1998; Khalid et al. 2004) and fungi (Frankenberger and Poth 1987). El-Abyad et al. (1994) reported that the addition of L-TRP to a nutrient medium significantly increased the production of IPYA by *Streptomyces griseoflavus*. Srinivasan et al. (1996) reported that some *Bacillus* spp. produced significant amounts of IAA when grown in a liquid culture medium supplemented with L-TRP, whereas less IAA was produced in a culture medium not supplemented with L-TRP. Our work supports these observations where we found that the concentrations of IAA and IPYA produced by *W. saturnus* were significantly higher in the L-TRP-amended medium compared to L-TRP-non-amended medium.

Response of plants to L-TRP alone in the absence of *W. saturnus* or *R. glutinis* could be attributed to the production of IAA by the resident microorganisms. PGRs biosynthesis in soil by rhizosphere microorganisms may increase upon the addition of their precursors. Nieto and Frankenberger (1989) reported that microbial biosynthesis of cytokinins was enhanced after the application of cytokinin precursors (adenine and isopentyl alcohol) to soil. Sarwar and Frankenberger (1994) also reported that the microbial biosynthesis of auxins was enhanced by L-TRP application to soil, resulting in enhanced maize growth.

Seedling inoculation with *W. saturnus* in the presence or absence of L-TRP resulted in a significant increase in the levels of endogenous IAA and IPYA in both roots and shoots, compared to *R. glutinis*-inoculated seedlings grown in L-TRP-amended or L-TRP-non-amended soil. It is noteworthy that the levels of endogenous auxins detected in the roots and shoots showed the same trend of responses as growth promotion resulting from the same treatments. This may also reflect that growth promotion observed may be related to auxin production. The endogenous auxins in the tissues assayed in *W. saturnus*-inoculated seedlings in the presence of L-TRP could also include auxins produced endogenously by the plants and/or the endophytic yeast and auxins absorbed by the roots from those produced by the indigenous microflora in the soil. Increases in the levels of endogenous auxins in treated plants after the application of IAA-producing rhizosphere bacteria have been reported. Fallik et al. (1989) reported that maize roots inoculated with IAA-producing isolate of *Azospirillum brasilense* have higher amounts of both free and bound IAA as compared to non-inoculated control, and the isolate also significantly increased root surface area compared to control. Other rhizosphere bacteria have been reported to produce IAA in vitro and to increase the amounts of IAA in treated plants (Prikryl et al. 1985).

In our study, IAA was detected in the culture filtrates of *W. saturnus* with or without amendment with L-TRP. The reports on the ability of the endophytic yeast *P. spartinae* (Nakamura et al. 1991) and the endophytic growth-promoting bacteria *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastian et al. 1998) to produce auxins in chemically defined culture media in the absence of L-TRP may explain the growth promotion obtained in our study in treatment 5 where the IAA-producing isolate of *W. saturnus* was not combined with exogenous supply of L-TRP.

There was a significant difference between the growth of maize seedlings inoculated with IAA-producing isolate of *W. saturnus* and its control in the absence of L-TRP. This does not correspond to the results obtained by Frankenberger and Poth (1987), who reported a non-significant difference in growth between Douglas fir trees inoculated with IAA-producing isolate of *Pisolithus tinctorius* and non-inoculated plants in the absence of L-TRP; however, the addition of a dilute solution of L-TRP with *P. tinctorius* produced a dramatic growth response. Our observations are not similar and indicate that our endophytic yeast isolate of *W. saturnus*, in the absence of L-TRP, was capable of obtaining the precursor from the host plant to synthesise auxins or was able to use pathways in plant tissues other than that involving L-TRP pathway. It is reported that certain bacteria can synthesise auxins from the precursor anthranilate without proceeding through L-TRP as an intermediate (Normanly et al. 1993; Zakharova et al. 1999). It is also possible that the differences in the responses reported could be related to the host species used in the two studies.

None of the studies to date, however, has involved inoculation of plant roots by endophytic yeasts; thus, this study is not only the first study involving root inoculation with endophytic yeasts but also one that has monitored root colonization by these yeasts for up to 8 weeks and related it to plant growth promotion. It is noteworthy that the isolate of *R. glutinis*, although not a plant growth promoter or an IAA producer, was a successful colonizer of roots up to 8 weeks of observation. This indicates that a yeast can colonize and multiply within root tissues without showing any obvious symptoms of growth promotion or deleterious effects. This study also indicates that there is a clear need to include plant growth-promoting endophytic yeasts in programs aimed at the use of microorganisms to enhance plant productivity at a field scale.

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