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CARFAX

A Rapid Microplate Callus Bioassay for Assessment of Rhizobacteria for Biocontrol of Leafy Spurge (*Euphorbia esula* L.)

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*Screening large collections of microorganisms for potential biological control activity on economically important weeds is often difficult. Seeds required as indicator species are often not available in large supply and are highly variable in viability and germination, resulting in uneven seedling development. A bioassay system for rapidly assessing the phytotoxic effects of rhizobacteria was developed based on leafy spurge (*Euphorbia esula L.*) callus tissue culture in multiple-well plates. Callus pieces (0.5 g) were placed in 24-well plates containing Gamborg's B5 medium, inoculated with rhizobacterial suspension and incubated at 27°C for 48 h. By rating inoculated callus for cellular damage, about 30% of rhizobacteria isolated from weedy* Euphorbia *spp. collected in Europe and North America were identified as being highly phytotoxic. Symptoms of phytotoxicity included growth reduction, discoloration and extensive cellular leakage. A high proportion of isolates found to be phytotoxic in tissue culture bioassays were similarly effective in standard leafy spurge seedling bioassays. The method is rapid, host specific and more uniform compared with assays using seedlings, and should be adaptable to other weed species for screening microorganisms for potential biocontrol activity.*

Keywords: *biological weed control, callus tissue culture,* Euphorbia esula, *leafy spurge, rhizosphere bacteria, weed management*

INTRODUCTION

Recent concerns about the effects of intensive pesticide use on environmental quality have led to an interest in sustainable agriculture systems with emphasis on reduced reliance on pesticides. As herbicides are reduced, other non-chemical control and management techniques must be developed to maintain weed infestations at economically low densities. A potentially effective alternative is the development of biological control technology. Recent reviews of the successful use of microorganisms in controlling certain weeds in

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specific agro-ecosystems (Charudattan, 1991; Kennedy & Kremer, 1996; Aldrich & Kremer, 1997) indicate the potential benefits. While fungal plant pathogens have been widely researched as biocontrol agents for weeds, deleterious rhizobacteria (DRB) that selectively suppress seedling growth of several weeds without affecting crop plants have been recently investigated and offer a new and effective biological control strategy for weed management (Kennedy & Kremer, 1996; Kremer & Kennedy, 1996).

The identification of microorganisms that are highly effective in the biological control of economically important weeds requires rapid and inexpensive bioassay systems with which to screen large numbers of isolates efficiently for potential bioactivity. The numbers of microbial strains tested in greenhouse and growth chamber conditions are limited due to the large amounts of space, supplies of materials and time-consuming steps required for properly replicated trials (Kloepper *et al.*, 1988). Seedling bioassays have been designed as alternatives to assess the growth of plants treated with selected fungal pathogens (Hoagland, 1995) or with DRB (Alstrom, 1987). Recently, digital image analysis of weed seedling roots treated with rhizobacteria has been developed in order to assess inhibitory activity rapidly and accurately and to reduce the tedious and time-consuming task of manually measuring root lengths in seedling bioassays (Doty *et al.*, 1994).

Although seedling root bioassays are routinely used to identify potentially inhibitory rhizobacteria (Alstrom, 1987; Doty *et al.*, 1994), the screening of certain weeds remains difficult because their seeds are not available in large supply and are highly variable in terms of viability and germination, resulting in uneven seedling development (Souissi & Kremer, 1994). Plant cell cultures are suitable systems with which to test the biological activity of agrochemicals, microbial toxins and allelochemicals, and the results are representative of those obtained in whole plant experiments (Mumma & Davidonis, 1983; Hogan & Manners, 1990; Song *et al.*, 1994).

DRB, which are non-parasitic bacteria that readily colonize root surfaces and suppress weed growth, are potentially effective biological control agents for many economically important weeds (Kremer & Kennedy, 1996). Several rhizobacteria have been reported to have high potential as biocontrol agents on leafy spurge (*Euphorbia esula*) (Souissi & Kremer, 1994). Leafy spurge is an aggressive perennial weed infesting over 1 million ha of rangeland in the northcentral US and prairie provinces of Canada (Pemberton, 1995). Long-term control with chemical herbicides is extremely difficult to achieve, emphasizing the critical need to develop effective biocontrol agents for use in a leafy spurge management programme.

The present authors previously demonstrated that leafy spurge cell culture suspensions can be used to assess detrimental effects of rhizobacteria (Souissi & Kremer, 1994). Although the cell suspension method was more reliable than seedling assays for detecting DRB, it was not suitable for screening large collections of bacteria because the cells (in 125-ml flasks) required controlled incubation in a water bath, harvesting by filtration and treatment by vital staining to determine the effects of bacterial inoculation. The objective of this study was to develop a microplate assay using leafy spurge callus maintained in microtiter plates in order to screen large collections of rhizobacteria rapidly and to identify those with the ability to suppress leafy spurge growth.

MATERIALS AND METHODS

Bacterial Inoculum Preparation

Rhizobacteria were isolated as previously described (Souissi & Kremer, 1994) from the rhizospheres and root homogenates of weedy *Euphorbia* spp. collected in Europe and North America. Representative colonies were purified and maintained on King's B medium (Sands & Rovira, 1970) and stored at 4°C until use. Bacterial inocula were prepared in phosphate-buffered saline (PBS) (10 mM-K_2PO_4–KH_2PO_4. 0.14 M-NaCl; pH 7.2) from 48-h-old cultures and adjusted to a concentration of 10^8 colony-forming units (CFU)/ml. Those

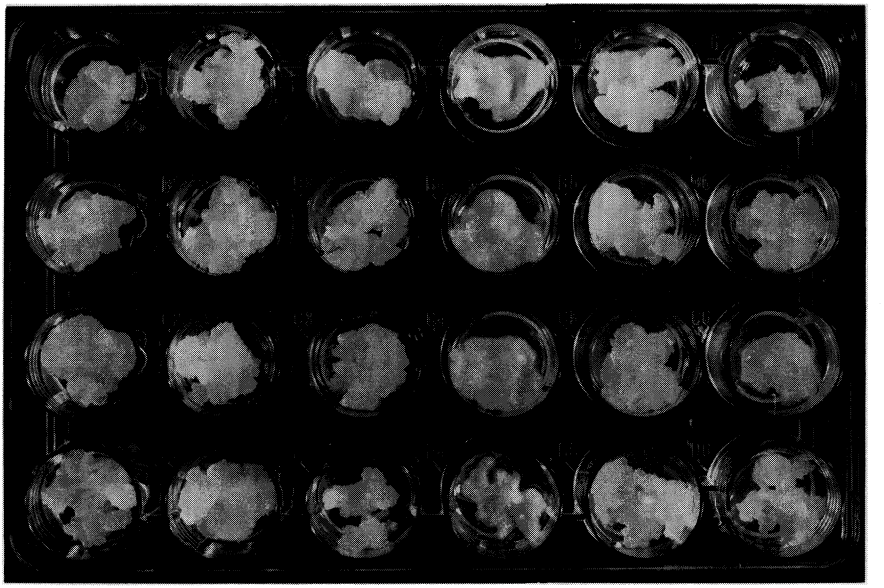

FIGURE 1. Microtiter plate bioassay for phytotoxicity of DRB isolates on leafy spurge callus. All wells initially contained about 0.5 g of fresh callus. The wells in the left lane (vertical column, lane1) are controls (not inoculated). The wells in the remaining lanes contain callus inoculated with selected DRB and incubated for 48 h. Note the liquid and callus disintegration in all wells of lane 4 (rating = 4) and no apparent effect on control callus or on inoculated callus in lane 2 (rating = 0). The ratings of inoculated callus in the remaining lanes range from 3.0 to 3.5.

rhizobacterial isolates used extensively in developing the callus tissue bioassay were identified using API/NFT test kits (bioMérieux Vitek Inc., Hazelwood, MO, USA) as described previously (Souissi & Kremer, 1994).

Development of Callus Bioassay

Leafy spurge callus tissue was grown and maintained on Gamborg's B5 medium (Gamborg *et al.*, 1968) supplemented with 1 mg l^{-1} of 2,4-D at 27°C and subcultured every 3 weeks. Callus pieces (0.5 g) were placed in each well of 24-well microtiter plates (Nunc InterMed, Roskilde, Denmark) containing Gamborg's B5 medium (Figure 1). Each callus piece was inoculated with 30 μl of a bacterial suspension, previously adjusted tubidimetrically to 10^8 CFU/ml at A_{600}. Plates were incubated for 48 h in the dark at 27°C. After incubation, each callus piece was visually rated for the appearance of injury due to inoculation on a 0–4 scale based on tissue discoloration, growth reduction and tissue disintegration (Figure 1 and Table 1).

TABLE 1. Description of ratings used in evaluating callus damage by rhizobacteria inoculated on leafy spurge callus cultured on Gamborg's B5 medium in microtiter plates. Callus was rated 48 h after incubation in darkness at 27°C

Rating	Description
0	No discoloration; no visible damage; no growth reduction
1	Slight discoloration; no visible growth reduction
2	Tissue color change; slight growth reduction
3	Tissue color change; obvious growth reduction, frequently manifested by 'tissue shrinkage'
4	Tissue color change; cellular leakage; callus disintegration; severe growth reduction

To validate the visual ratings of tissue damage, cell viability was quantitatively assessed using the Evans blue assay (Atkinson *et al.*, 1990), modified as previously described (Souissi & Kremer, 1994). After incubation, inoculated callus pieces were resuspended and incubated in 0.5% aqueous Evans blue reagent for 45 min and washed in deionized water. The dye was extracted from cells by macerating each callus piece in 1 ml of deionized water using a Stomacher Lab-Blender (Tekmar, Cincinnati, OH, USA). The absorbance of the supernatant, determined at 630 nm, increased with the proportion of non-viable cells. Several rhizobacterial isolates that induced different levels of tissue damage were selected for bioassays to correlate the visual rating system with cell viability determined by the Evans blue assay method.

To quantify the effects of DRB on tissue growth, callus pieces were inoculated with different DRB isolates, removed from microplates periodically and weighed. The fresh weights of callus pieces were compared with those not receiving DRB and incubated for the same periods of time. Callus fresh weights were expressed as the percentage of the non-inoculated controls.

The effects of DRB cell density on callus growth were determined by inoculating callus pieces with different cell concentrations of selected DRB-inducing callus ratings of 3.5–4.0 and determining the callus fresh weight 48 h after inoculation. Similarly, the growth response of callus to different bacterial cell concentrations over time was evaluated by inoculating callus pieces with appropriate cell densities and recording the growth every 24 h for 5 days. For both cell density experiments, callus fresh weights were expressed as the percentage of non-inoculated control callus pieces.

Seedling Bioassay

To determine the relationship between callus rating and the deleterious effect of rhizobacteria, selected DRB were assayed on lettuce (*Lactuca sativa* L. cv. Blackseeded Simpson) and leafy spurge (Montana Accession) using standard seedling bioassays on 1.0% agar. Lettuce is a standard indicator species for plant growth responses to rhizobacteria (Alstrom, 1987). The root lengths of seedlings were recorded 48 h after inoculation.

Statistical Analysis

All tissue culture and seedling bioassay studies were based on a complete random design with four replicates and were repeated at least once. Data were subjected to regression analyses and analysis of variance (ANOVA) procedures. Where F-values were significant at the $P < 0.05$ level, the means were compared using Fisher's protected least significant difference (LSD) test. Data from callus tissue growth response experiments were averaged and variation was expressed as standard error.

RESULTS AND DISCUSSION

The bioassay developed was able to differentiate between rhizobacteria with the highest growth inhibitory effect and those with moderate or no effect on callus growth. Callus pieces in microtiter plate wells observed 48 h after inoculation with growth-inhibitory rhizobacteria showed various levels of damage, including browning, cellular leakage and, in the most severe cases, complete tissue maceration (Figure 1). The variation in symptoms caused by the rhizobacteria suggests differences in modes of action and in the biochemistry of the plant–rhizobacteria interactions in callus tissue (Goodman *et al.*, 1986). The callus rating system developed based on these symptoms provided a good measure of callus sensitivity to the deleterious effects of various rhizobacteria (Table 1).

Although the development of the rating system was based on several parameters, the measurement of fresh weight reduction was the most reliable and the most consistent way to differentiate between rhizobacteria with various effects. Rhizobacteria with the highest inhibitory effects resulted in a significant reduction in callus fresh weight accumulation 48 h

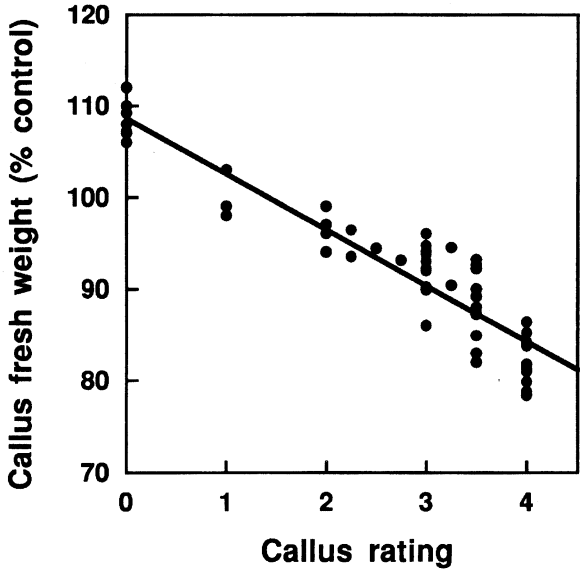

FIGURE 2. The relationship of callus rating at 48 h of incubation with various DRB isolates as a function of callus growth reduction. The regression equation is $y = -6.09x + 108.64$.

after inoculation compared with controls (Figure 2). Regression analysis indicated a strong linear relationship ($R^2 = 0.865$) between callus fresh weight and callus rating. This suggested that visual ratings of callus in microtiter plates could be used to estimate decreases in fresh weights accurately.

Quantitative analysis of the differential responses of calli exposed to various rhizobacteria using the Evans blue bioassay indicated a positive linear relationship ($R^2 = 0.942$) between the A_{630} of callus extracts and the visual ratings of calli treated with rhizobacteria (Figure 3). This result confirmed that visual ratings of callus growth were as accurate as quantitative cell viability measurements based on A_{630} of callus extracts in detecting callus sensitivity to the phytotoxic effects of DRB.

While the fresh weight of callus receiving no inocula increased by 40% over the 120-h incubation period, the fresh weight of callus receiving various cell concentrations of the selected DRB *Pseudomonas syringae* isolate 8-1 was significantly reduced (Figure 4). A reduction in callus weights was observed even at low bacterial concentrations. This indicated that the growth-inhibitory activity of DRB may occur at low levels of the rhizobacterial population. However, callus weight reduction was greatest at high bacterial concentrations during the first 48 h, indicating that short incubation periods caused substantial growth inhibition.

Callus fresh weight reduction was proportional to increasing cell concentrations of DRB (Figure 5). Although all selected DRB reduced growth when inoculated to calli at different concentrations, growth reduction was greater with *P. syringae* isolate 8-1 at all concentrations compared with the other isolates tested. This indicated the sensitivity of the bioassay, with an ability to distinguish between DRB with different levels of phytotoxicity towards leafy spurge callus. In general, high cell concentrations (10^8 cells/ml) of all isolates tested damaged callus tissue within 24–48 h; even lower concentrations caused damage after 48 h. Other bioassays using seedlings as indicators of microbial phytotoxicity have shown similar patterns of differential sensitivity among isolates of fungal pathogens (Hoagland, 1995) and rhizobacteria (Kennedy *et al.*, 1991) undergoing evaluation.

The phytotoxic effects of DRB using the callus rating system did not correlate with the

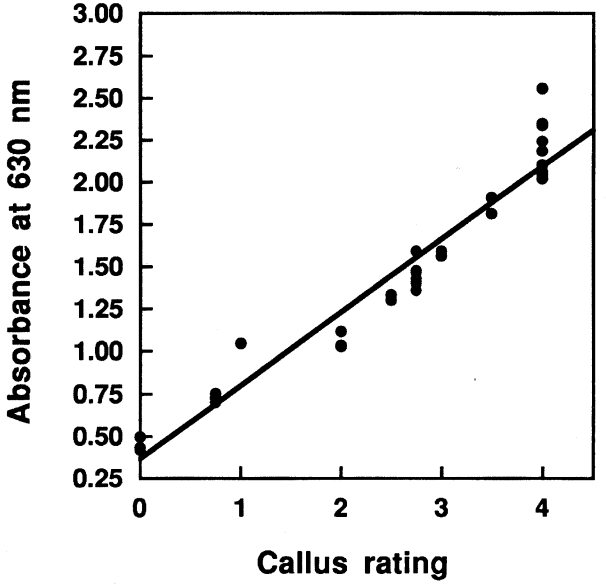

FIGURE 3. The relationship between leafy spurge callus rating and cell viability determined by the Evans blue method. Callus was inoculated with DRB isolates and incubated for 48 h. Cell viability is indirectly proportional to absorbance at 630 nm. The regression equation is $y = 0.43x + 0.36$.

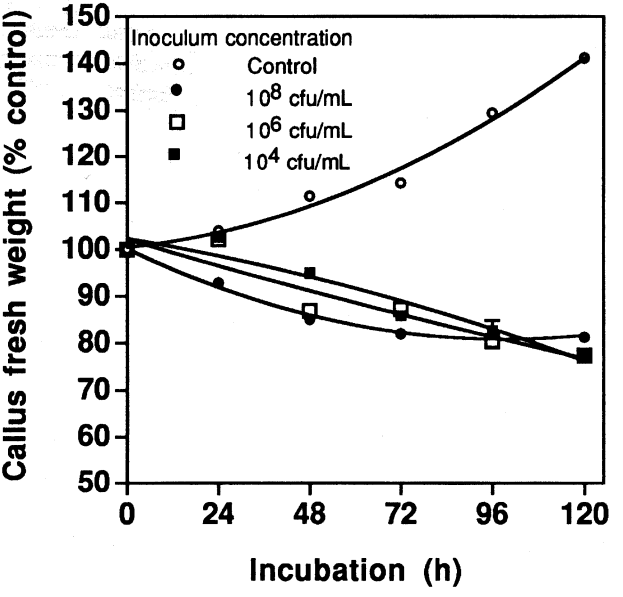

FIGURE 4. The effect of cell concentrations of *P. syringae* pv. *syringae* isolate 8-1 on the growth of leafy spurge callus incubated in the dark. Vertical bars indicate the standard error of the mean; bars not visible are hidden by the plot symbol. The regression equations are: control, $y = 0.002x^2 + 0.079x + 100.48$; 10^4 CFU/ml, $y = -0.001x^2 - 0.14x + 102.34$; 10^6 CFU/ml, $y = -0.024x + 102.21$; 10^8 CFU/ml, $y = 0.002x^2 - 0.39x + 100.2$

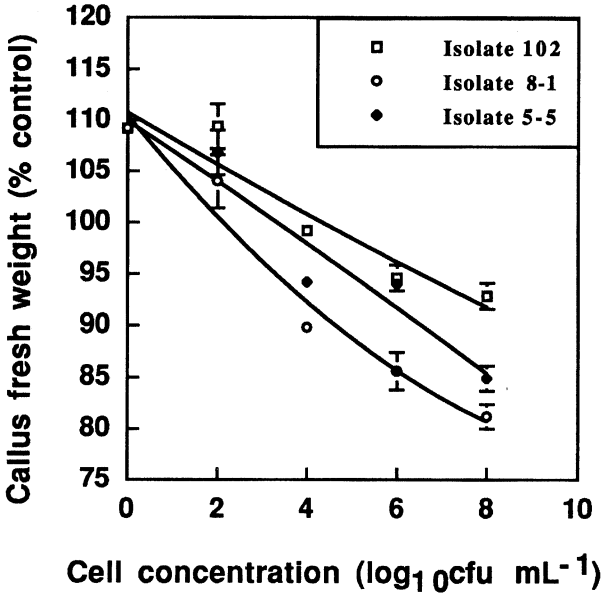

FIGURE 5. The effects of various cell concentrations of selected DRB isolates (*P. fluorescens* 102, *P. fluorescens* 5-5 and *P. syringae* pv. *syringae* 8-1) on fresh weight accumulation of leafy spurge callus incubated in the dark for 48 h. Vertical bars indicate the standard error of the mean; bars not visible are hidden by the plot symbol. The regression equations are: isolate 102, $y = 0.03x^2 - 2.62x + 110.8$; isolate 5-5, $y = -0.018x^2 - 2.92x + 109.96$; isolate 8-1, $y = 0.20x^2 - 5.38x + 110.49$.

inhibition of root growth using lettuce seedling bioassays (Figure 6(a)). Even though lettuce seedlings are often used in preliminary evaluations of phytotoxicity (Alstrom, 1987), the lack of a good correlation with callus ratings suggests that the inhibitory activity of the majority of DRB is due to plant host and, in this study, is more specific to leafy spurge. Previous work has also shown that the inhibitory activity of rhizobacteria originating from different grasses depended on plant species (Doty *et al.*, 1994). A comparison of phytotoxicity detected by the callus bioassay and a leafy spurge seedling bioassay using several different DRB revealed a good correlation between these two methods (Figure 6(b)). Highly phytotoxic rhizobacteria yielded callus damage ratings of 3–4 and significantly reduced leafy spurge seedling root growth by 45–92%. Similarly, bioassays for phytotoxicity of the fungal pathogen *Fusarium solani* revealed that growth reduction of soya bean (*Glycine max* (L) Merr.) seedlings inoculated with the pathogen significantly correlated with sensitivity of soya bean callus to culture filtrates of the pathogen (Jin *et al.*, 1996).

The practical application of the callus bioassay system was demonstrated by screening a large collection of rhizobacteria (more than 2000 isolates) from *Euphorbia spp.* collected in Europe and North America (Figure 7). About 30% of the rhizobacteria were highly phytotoxic. This proportion of potential phytotoxic isolates is in general agreement with that reported for bacterial collections screened using other bioassay methods (Kremer *et al.*, 1990). Isolates will be tested under greenhouse and field conditions to verify the accuracy of the callus bioassay system.

The microplate callus bioassay and leafy spurge seedling bioassay detected inhibitory activity by the tested isolates (Figure 6(b)). The callus rating method was about 20 times faster than the seedling root measurement method, with each isolate requiring 30 s for callus rating or 10 min for the seedling root method. The microplate callus bioassay required considerably less incubation space, since one microtiter plate accommodating 24 tests is

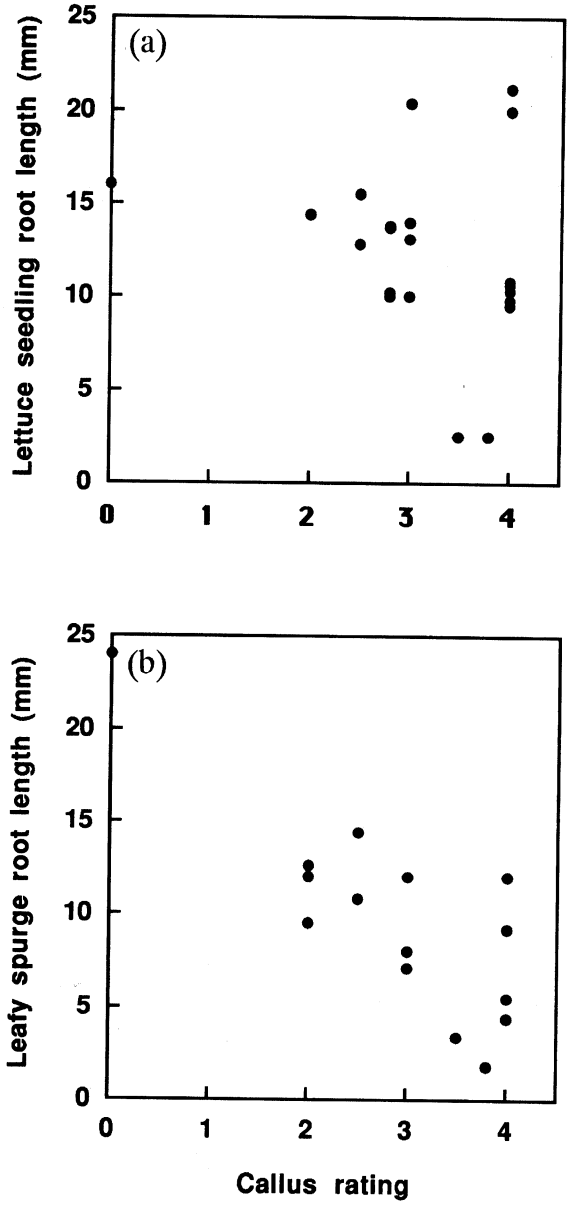

FIGURE 6. The relationship between seedling root growth of lettuce (a) and leafy spurge (b) with leafy spurge callus ratings after inoculation with selected DRB isolates. Root lengths of lettuce and leafy spurge seedlings were determined at 48 and 96 h respectively after inoculation; callus ratings were determined 48 h after inoculation.

equivalent to 24 Petri plates required by the seedling bioassay in an identical trial. Thus, the callus rating method provides a rapid presumptive assessment for phytotoxic activity when surveying thousands of rhizobacterial isolates for potential biocontrol activity. Since callus showed differential sensitivity to the several isolates tested, the callus bioassay system using microtiter plates has potential for rapid screening of modes of action within a

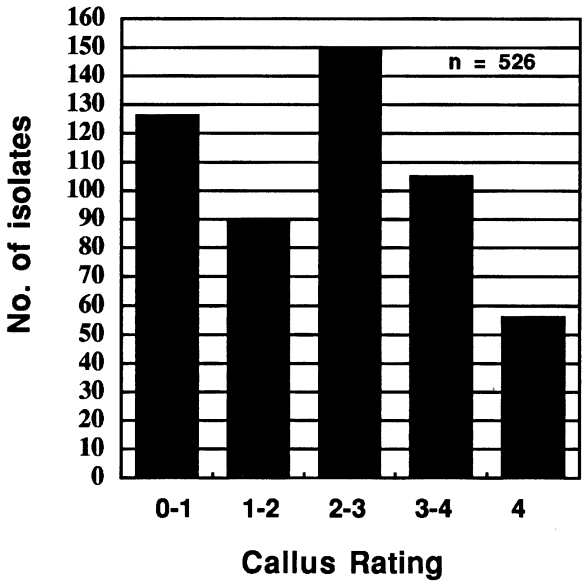

FIGURE 7. Distribution of DRB isolates from leafy spurge based on ratings obtained using the callus tissue bioassay system in microtiter plates.

collection of different microorganism or for screening microbial metabolites that may affect leafy spurge growth. The method should be easily adapted for screening DRB on other weeds targeted for biocontrol provided that tissue culture can be developed for the weed hosts.

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