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Induction of Leafy Galls in *Acacia mearnsii* De Wild Seedlings Infected by *Rhodococcus fascians*

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ABSTRACT

*Plantlets of blackwattle (*Acacia mearnsii *De Wild) were inoculated with the bacterium* Rhodococcus fascians *and cultured in vitro. Leafy galls appeared at the cotyledonary nodes in 75% of the infected plants. The galls were separated from the plants and cultured on a medium containing three-quarters-strength MS salts (Murashige and Skoog, 1962), MS vitamins, 2% sucrose and an antibiotic (cephalothin), supplemented with or without 0.2% activated charcoal. Histological studies conducted from the sixth to the twenty-second day after plant infection revealed the presence of newly formed meristematic centers, first in the axillary region, then on the petioles and lamina of the leaflets around the apical meristem. Approximately 37% of the galls developed one shoot with both concentrations of cephalothin.*

Key words: Black wattle, legume tree, micropropagation

INTRODUCTION

Rhodococcus fascians (Tilford) is a Gram-positive bacterium (actinomycetes) which infects a great array of dicotyledonous and monocotyledonous species, resulting in the induction of malformations. The most typical is the leafy gall, formed by multiple meristematic centers and short hypertrophied shoots in which elongation is suppressed (Vereecke et al., 2000).

In the case of strain D188, the induction of fasciation is related to the presence of a linear plasmid of 185 kb, called pFiD188, carrying several loci involved in gall formation (Desomer et al., 1988; Crespi et al., 1992, 1994; Stange et al., 1996). A mutation in the locus *att* induced an attenuated fasciation, producing small galls. A mutation in the locus *hyp* provokes an hyperfasciation leading to the formation of bigger galls, when compared with wild type (Crespi et al., 1994). The *fas* locus, essential for pathogenicity, is involved

in the synthesis of the main virulence factor. It contains a gene (*fasD*) showing some homology with the isopentenyl adenine (*ipt*) genes of *Agrobacterium tumefaciens* and *Pseudomonas* species. The *ipt* gene codifies the enzyme that catalyses the first and rate-limiting step in the synthesis of cytokinin, the formation of isopentenyl adenine from 5' AMP and dimethylallyl pyrophosphate (Crespi et al., 1992). However, some experimental data suggest that the function of the *fasD* gene is distinct from the production of classical cytokinins. On the other hand, auxin levels are also modified in plants carrying a leafy gall, leading to a modification of the cytokinin/auxin ratio (Goethals et al., 2001).

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The use of *Rhodococcus fascians* for gall formation may provide a new way for bud regeneration, as it allows the proliferation of buds from meristematic tissues, without the use of growth regulators. This property was demonstrated by Goethals et al. (1998) for a wide range of plant species. Subsequently, such buds can be separated and induced to elongate and form roots, and may provide a new method for micropropagation.

Acacia mearnsii is a legume tree of great economic importance in the south of Brazil. It is difficult to propagate vegetatively and by classical micropropagation techniques (Quoirin et al., 2001). The proliferation of buds induced by *Rhodococcus fascians* should help the *in vitro* multiplication of this tree species, avoiding the use of expensive growth regulators. Moreover, it could be possible to implement a procedure for genetic transformation by inoculating the plantlets with *Agrobacterium tumefaciens* carrying foreing genes. The inoculation with *A. tumefaciens* and *Rhodococcus fascians* could be done at the same time or at different moments. The objective of the present work was to establish the conditions for gall induction in blackwattle (*Acacia mearnsii*) seedlings and study the anatomy of the leafy galls.

MATERIALS AND METHODS

Plant material

Seeds of *Acacia mearnsii* were obtained from TANAGRO (Rio Grande do Sul, Brazil). The seeds were scarified for one minute in boiling water, disinfected in commercial sodium hypochlorite (5-6%) for 10 min and rinsed three times in sterile water. The seeds were germinated in sterile flasks (60 mm dia., 80 mm high) on cotton soaked with half-strength MS mineral solution. After that, they were incubated at 24 ± 2° C, under fluorescent daylight tubes (approximately 40 $\mu mol.m^{-2}.s^{-1}$), with a photoperiod of 16 h.

Bacterial strains - The strains D188 and D188-5 of *Rhodococcus fascians* (Desomer et al., 1988) were obtained from the Institute of Genetics, University of Ghent, Belgium. The D188-5 strain is not pathogenic and does not induce gall formation. It was used as a control. The bacteria were grown in solid yeast extract broth (YEB) medium (Miller, 1972) for 24h, then transferred into 20 mL of liquid YEB medium and shaken at 28° C for 24h.

Infection

One week after germination, seedlings with expanded cotyledonary leaves and the first true leaf were inoculated with the bacteria. They were submerged in the bacterial culture for 45 to 60 min and then vacuum infiltrated for 5 min.

Tissue culture

After inoculation, the seedlings were cultured on agar-solidified medium containing MS mineral salts and organic compounds, supplemented with 2.9 or 29.2 mM sucrose. The trial was repeated three times. The formation of gall and plantlet necrosis were evaluated. The flasks containing the seedlings were incubated under the conditions previously described. Approximately 40 days after infection, the galls were separated from the plantlets and transferred into a culture medium containing three-quarters-strength MS mineral solution (MS3/4), MS vitamins and 58.5 mM sucrose. First, AC was omitted in the culture medium and cephalothin (cephalosporin) was added at 500 $mg.L^{-1}$. This trial was repeated five times and the formation of galls was evaluated. In another series of nine trials, 0.2% (w/v) activated charcoal (AC) was added to the media supplemented with cephalothin (250 or 500 $mg.L^{-1}$). Some of the cultures were maintained in the dark and others under a photoperiod of 16 h. The development of shoots was evaluated in the plants receiving light. After 15 days on the culture medium, part of the galls which did not produce shoots were transferred to the same medium, to which 0.5 or 2 $mg.L^{-1}$ of filter sterilized gibberellic acid (GA_3) was added after medium autoclaving.

Histological techniques

Samples were collected every three days, from the fifth day after infection until the 30th day. The apical region was fixed in formaldehyde/acetic acid/alcohol 70% (v/v) (Johansen, 1940) under vacuum for 24 h. After that, the material was transferred into 70% (v/v) ethanol and dehydrated in an ethanol series. The samples were then embedded in glycolmethacrylate (GMA) following the procedure of Feder and O'Brien (1968) and the manufacturer´s instructions. Longitudinal sections 5 to 7 μm thick were made with a rotary microtome. They were stained with toluidine blue (O'Brien et al., 1965) and mounted in Entelan. Sections were also made of control plants either inoculated with strain D188-5 or not inoculated.

The tissues were photographed with a Zeiss Axiofot microscope.

RESULTS

Gall formation

The leafy galls were observed macroscopically 10 days after infection (Figs. 1a and b). They developed above the cotyledonary node, in the region of the axillary buds and caulinar apex. The percentage of plants forming leafy galls was 78.2% when medium contained 2.9 mM sucrose and 56.2% with 29.2 mM sucrose (Table 1). During this stage, the percentage of dead seedlings was 4.1% when medium contained 2.9 mM sucrose and 22.9% when it contained 29.2 mM sucrose. The seedlings inoculated with strain D188-5 did not form galls. These results indicated that a low concentration of sucrose in the culture medium was more favorable to gall formation than a concentration of 29.2 mM. In subsequent experiments, the 2.9 mM sucrose concentration was used and the percentage of seedlings forming a leafy gall was maintained around 75%.

Leafy galls separated from plantlets were cultured in the presence of an antibiotic, in order to inhibit the growth of the bacteria and induce the formation of shoots. Table 2 indicates the percentages of galls forming shoots when the media contained cephalothin without AC. The formation of shoots was observed in 23.3% of the galls and 10.3% of them became necrotic (data not shown). When media contained charcoal and two concentrations of cephalotin were compared, these percentages varied from experiment to experiment (Table 3). There were a few differences between the results obtained with the two concentrations of cephalothin and the mean percentage calculated for both concentrations of cephalothin was 37%. In all cases, only one shoot developed from each gall and 25% of the galls became necrotic and died (data not shown). In an attempt to better kill the bacteria and to favor shoot development, the galls were immersed in solutions of cephalothin (100 $mg.L^{-1}$ for 10 min or 200 $mg.L^{-1}$ for 20 min) before their transfer into the culture medium. These treatments did not raise the number of galls with shoots.

Table 1 - Effect of sucrose concentration in the culture medium on the induction of leafy galls in *A. mearnsii* seedlings, recorded 6 weeks after inoculation with *Rhodococcus fascians* (three trials).

Trials	Percentage of seedings forming leafy galls (*)		Percentage of dead seedlings	
	Sucrose concentration (mM)		Sucrose conc. (mM)	
	2.9	**29.2**	**2.9**	**29.2**
1	80	36.4	0	25.0
2	75	70.8	4.0	4.2
3	79.5	61.5	8.3	39.5
Mean±SD	**78.2 ± 2.7**	**56.2 ± 17.8**	**4.1**	**22.9**
VC %	3.52	28.93		

*Number of seedlings with gall/ number of inoculated seedlings minus dead seedlings
VC: variation coefficient

When the galls were maintained in the dark, all of them became necrotic after three weeks. The galls transferred into the media containing GA_3 and AC did not form shoots.

Histological studies

In the control plant of *Acacia mearnsii* (Fig. 1a), the apical meristem presented two layers of cells, constituting the tunica, and a group of cells situated internally, which form the corpus

(Fig. 1c). The apical meristem showed cells with dense cytoplasm and a large nucleus in the region of the tunica/corpus and procambium (Fig. 1c). When plantlets remained seven days in contact with *Rhodococcus fascians,* the apical meristem appeared darker and the cytoplasm of the cells was denser than in the control (Fig. 1d).

Table 2 - Shoot formation four weeks after transfer of leafy galls on culture medium containing MS3/4 and 500 mg.L^{-1} cephalothin (5 trials).

Trials	Percentage of galls forming shoots (*)
1	0
2	11.1
3	50.0
4	34.8
5	20.8
Mean ± SD	**23.3 ± 19.6**
VC %	84.15

* Number of galls with shoot/total number of galls minus dead galls
VC: variation coefficient

Table 3 - Shoot formation four weeks after transfer of leafy galls on culture medium containing MS3/4, 0.2% (w/v) activated charcoal and two concentrations of cephalothin (9 trials).

Trials	Percentage of galls forming shoots in the presence of cephalothin (*)	
	250 mg.L^{-1}	**500 mg.L^{-1}**
1	22.2	3.3
2	9.5	30
3	42.8	30.8
4	62.8	38.9
5	41.7	23.1
6	66.7	28.6
7	0	72.7
8	66.7	37.5
9	61.5	33.0
Mean±SD	**41.5 ± 25,60**	**33.1± 21,11**
VC %	61,6	63,8

* Number of galls with shoot/total number of galls minus dead galls
VC: variation coefficient

Near the apical meristem, new meristematic centers were observed, primarily in the axils of the plantlets (Fig. 1d) and, after eleven days, on the lamina and petioles of the leaflets (Figs. 1g and h), showing a typical aspect when compared with the control plant (Fig. 1e). Twelve days after inoculation, the bacteria were visible at the surface of the meristem (Fig. 1f). After 22 days, there was no difference in the aspect of the meristematic centers. When maintained on the same medium, the leafy galls formed 15 to 40 meristematic centers after three months.

DISCUSSION AND CONCLUSION

The inoculation of *Acacia mearnsii* plantlets with *Rhodococcus fascians* was effective with regard to the formation of galls. This process was favored by a low concentration of sucrose in the culture medium. As the carbohydrates constitute a carbon source for the bacterium, its reduction in the medium may lower the growth of the bacterium at the medium surface and, as a consequence, the necrosis of the seedlings that we observed.

The variation among the results of gall formation induced by *R. fascians* was high, as was also observed for tumor formation when *Acacia* species

were inoculated with *Agrobacterium tumefaciens* wild strains (Quoirin et al., 2000).

The number of shoots developed from the galls was low and only one shoot developed in every gall. In addition, necrosis of the leaves occurred for approximately 25% of the galls. The lack of elongation could be due to the presence of the bacteria in the galls, even after their immersion in a solution of antibiotic. The bacteria seem to modify the hormonal balance of the tissues, which has already been demonstrated for geranium plants (Balázs and Sziráki, 1974). This inhibition of shoot outgrowth has been interpreted as an extreme form of apical dominance, as the shoot primordia present in development (Goethals et al., 2001).

Studies conducted by Vereecke et al. (2000) showed a great susceptibility to infection by *R. fascians* of plant species from 70 genera and 32 families. It was also noted that symptoms depended on several factors of the plant and of the bacteria. The plant reaction differed from one species to another, as for example *Nicotiana tabacum* and *Arabidopsis thaliana.* We observed the same differences between *Acacia mearnsii*, which formed leafy galls in the majority of the plantlets, and *Acacia mangium*, for which this formation was reduced (data not shown). In *Arabidopsis*, shoots are formed in the axils of leaves and not at the apical meristem (Vereecke et al., 2000). Each activated axillary bud generates multiple shoots, which are inhibited in their outgrowth and often misshapen (Ritsema, *in* Manes et al., 2001). We observed the same shoot formation in *Acacia mearnsii* axillary regions. In tobacco, axillary meristems were activated by *Rhodococcus fascians* infection, but there was also a dedifferentiation of tissues of the stem and leaves (Manes et al., 2001). Shoots originated both from existing meristematic tissues (axillary meristems) and from *de novo* initiated meristems in the petioles and veins of the leaves (Vereecke et al., 2000). As in the case of tobacco, the presence of meristematic regions was observed in the petiole and lamina of *A. mearnsii* leaflets. In *Atropa belladona,* the galls developed in the region of the apical meristem (Vereecke et al., 2000).

In *A. mearnsii,* the bacteria were present at the surface of meristematic centers, as was described for tobacco by Cornelis et al. (2001). However, Cornelis et al. (2001) also showed the presence of the bacteria in the intercellular spaces, a feature we did not observe in *A. mearnsii* tissues by optical microscopy.

Cornelis et al. (2001) demonstrated that the *fas* locus of plasmid pFD188 was expressed in the epidermis and, at a later stage of the infection, in the meristematic outgrowths developing into shoot primordia. This dedifferentiation of parenchymatous cells and proliferation of shoot meristems could be due to the effect of a cytokinin-like compound the synthesis of which was reported to be induced by the *fas* locus (Goethals et al., 1995).

The absence of response by the galls to the addition of GA_3 in the media could be due to the effect of AC, as this compound adsorbed growth regulators such as gibberellins (Pan and Van Staden, 1998). It could also be due to the fact that some bacteria remain in the galls, even after treatment with antibiotic.

In this work, we demonstrated that gall formation was induced in *Acacia mearnsii* plantlets inoculated with *Rhodococcus fascians*. These galls were formed of multiple buds which did not elongate, possibly due to the particular hormonal balance of the gall tissues. New conditions of culture, including medium composition and other antibiotic treatments, need to be tested in order to obtain better shoot formation from the galls. Once this step is improved, this method could constitute a source of multiple shoots suitable for plant propagation, with the advantage that no plant growth regulator would be necessary to induce this proliferation of buds.

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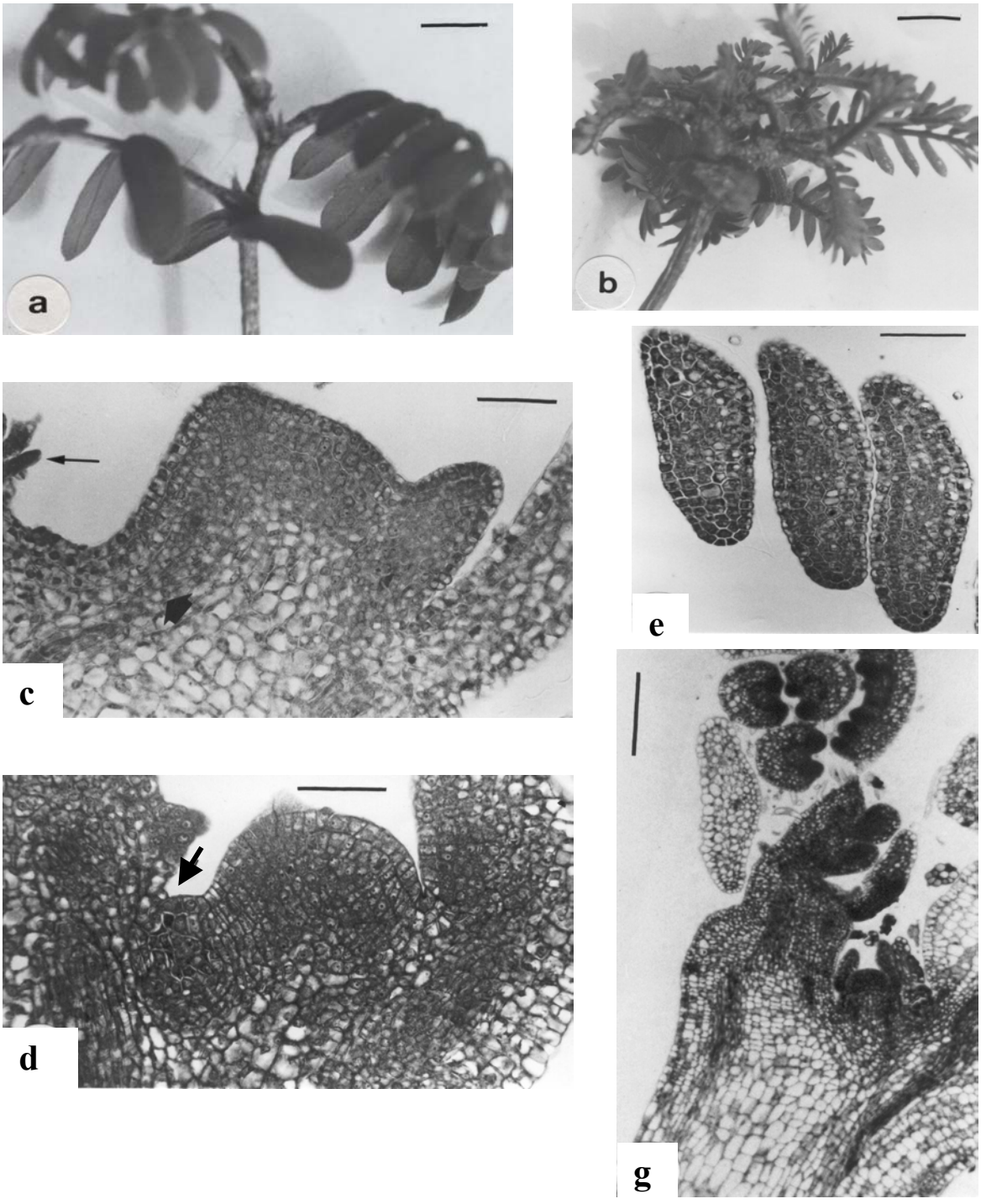
RESUMO

Plantas recém germinadas de acácia negra (*Acacia mearnsii* De Wild.) foram inoculadas com a bactéria *Rhodococcus fascians* e cultivadas *in vitro.* Galhas cobertas por folhas apareceram na

altura do nó cotiledonar em 75% das plantas infectadas. As galhas foram separadas das plantas e cultivadas num meio de cultura contendo os sais do meio MS (Murashige e Skoog, 1962) reduzidos a 3/4, as vitaminas do mesmo meio, 2% de sacarose e um antibiótico (cefalotina), adicionado ou não de 0,2% de carvão ativo. Estudos histológicos realizados entre o sexto e o vigésimo segundo dia depois da inoculação, revelaram a presença de centros meristemáticos novos, primeiro nas regiões axilares, em seguida nos pecíolos e limbos dos folíolos ao redor do meristema apical. Aproximadamente 37% das galhas desenvolveram um broto na presença de cefalotina.



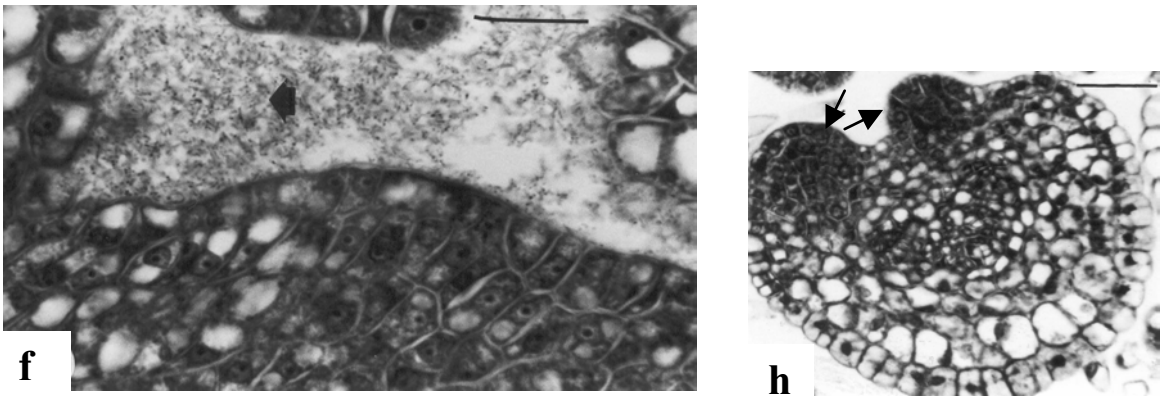


Figure 1 - *Acacia mearnsii* plantlets. (a) Control after 45 days. Bar: 3.6 cm. (b) Plantlet inoculated with *Rhodococcus fascians* after 45 days (bar: 3.6 cm). (c) Longitudinal section of caulinar apical meristem of control plantlet 27 days after germination (bar: 200 μm) (arrow: trichome, arrowhead: procambium). (d) Longitudinal section of caulinar apical meristem of infected plantlet 7 days after inoculation (arrow: axillary meristematic center) (bar: 500 μm). (e) Transversal section of the leaflets of control plants (bar: 500 μm). (f) Detail of meristem surface after twelve days (arrowhead: bacteria) (bar: 20 μm). (g) Longitudinal section of caulinar apex eleven days after inoculation (bar: 200 μm). (h) Detail of fig. g (arrows: meristematic centers in the petiole) (bar: 10 μm).

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