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# *In vitro* susceptibility of two tropical *Acacia* species to *Agrobacterium tumefaciens*

# Suscetibilidade à infecção por *Agrobacterium tumefaciens* de duas espécies tropicais de *Acacia in vitro*

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**ABSTRACT**: *Acacia mangium* and *A. mearnsii* are leguminous N-fixing trees which can be used for afforestation of degraded soils. The application of modern techniques of genetic transformation to such species should allow some traits, like insect resistance, to be improved, or to modify lignin metabolism. In order to establish an efficient transformation system for these species, ten wild strains of *Agrobacterium tumefaciens* were tested, using two methods of inoculation. Micropropagated shoots or tissues obtained from seedlings were inoculated. Several experimental conditions were compared: type of explant, concentration of the bacterial suspension and presence of acetosyringone. The frequency of tumour induction indicated variations between strains and types of plants, as well as an effect of bacterial concentration. The tumours were characterized by their hormone independent growth. Roots were formed at the wounding site or at the tumour base. Opines were not detected in the tumours analysed, indicating a weak expression of bacterial genes.

**KEYWORDS**: *Acacia mangium*, *Acacia mearnsii*, *Agrobacterium*, Legumes

**RESUMO**: *Acacia mangium* e *Acacia mearnsii* são árvores da família das Leguminosas que desenvolvem uma simbiose com bactérias fixadoras de nitrogênio. Graças a esta característica, elas podem ser utilizadas para reflorestamento de áreas degradadas. A aplicação de modernas técnicas de transformação genética a tais espécies poderia permitir melhorar certas características, como resistência a insetos, ou modificar o metabolismo da lignina. O objetivo do trabalho foi testar cepas selvagens de *Agrobacterium tumefaciens* a serem utilizadas num protocolo de transformação genética destas espécies. Dez cepas foram testadas, usando dois métodos de inoculação. Dois tipos de plantas foram inoculados: brotos de plantas micropropagadas e plantas recém-germinadas de um mês de idade. Várias condições experimentais foram comparadas: tipo de explante, concentração da solução bacteriana e presença de acetosiringona. A freqüência de indução de tumores indicou variações entre cepas bacterianas e tipos de plantas, assim como um efeito da concentração da bactéria no momento da inoculação. Os tumores foram caracterizados por seu crescimento independente da presença de hormônios. A formação de raízes foi observada na base do tumor ou no lugar do ferimento do caule. Opinas não foram detectadas nos tumores analisados, indicando uma expressão fraca dos genes bacterianos.

**PALAVRAS-CHAVE***: Acacia mangium, Acacia mearnsii, Agrobacterium,* Leguminosas

## INTRODUCTION

*Acacia mangium* and *Acacia mearnsii* are two legume species used in reforestation programs of low fertility soils. Their N-fixing ability contributes to satisfactory establishment and subsequent growth. *A. mangium* is native to Northeastern Australia and New Guinea. It naturally occurs at an altitude below 300 m in the humid tropical zone. It grows on acidic soils, often of low fertility and sometimes with restricted drainage. *A. mangium* has a good stem form, superior coppicing hability and great stability during storms. The timber of this species is suitable for general construction, furniture, particle board, veneer and paper pulp (National Research Council, 1983). *A. mearnsii,* also native to Australia, grows in the moist subtropical highlands (up to 2000 m) of the south of Brazil (Boland et al, 1984). This species is used for fuelwood, charcoal and leather tanning. Both species are important for reforestation in south and north of Brazil, especially where soils are damaged by intensive cultivation, mining or deforestation (Dias et al., 1991).

The introduction of new desirable traits in these species by classical breeding is delayed because of the long generation time. Gene transfer is essential to introduce novel genetic characters such as disease and insect resistance. This transfer can be mediated through *Agrobacterium* infection. *A. tumefaciens* is a soil Gram negative bacterium, which causes the crown gall disease, characterized by the presence of a tumor at the infection site. This bacterium introduces specific DNA sequencies into the plant genome (Chilton et al., 1977).

The host range of *Agrobacterium* is wide for dicotyledonous plants but restricted for monocotyledonous and gymnosperm species. However, susceptibility varies between species and even between varieties of a species. The virulence of the bacterium depends on the strain and its interaction with the host plant. In order to develop a strategy of genetic transformation of *Acacia* species through the use of *Agrobacterium*, it is important to determine the best host-pathogen combination for each species (Lacorte e Mansur, 1993). In case of the *Acacia* genus, Bray et al. (1994) described the production of calli after infection of cuttings of *A. flava* and *A. nilotica* with strain LBA4404. The purpose of this work was to determine the susceptibility of *A. mangium* and *A. mearnsii* plantlets to infection with several strains of *Agrobacterium tumefaciens* under *in vitro* conditions.

## MATERIAL AND METHODS

### *Plant material and tissue culture*

Plants of *A. mangium* were obtained from the Centro Nacional de Pesquisas de Agrobiologia, EMBRAPA (Rio de Janeiro, Brazil). They were micropropagated from axillary buds on multiplication medium consisting of MS salts (Murashige e Skoog, 1962) supplemented with 0.5 mg.$L^{-1}$ 6-benzyladenine, 2% sucrose and 0.75% Sigma® purified agar. The culture conditions were: temperature of 28 ± 4º C, mixed day light and Sylvania® Grolux fluorescent tubes, giving a light intensity of 40 µmol. $m^{-2}$ $s^{-1}$, and a photoperiod of 16h.

Seeds of *A. mangium* and *A. mearnsii* were provided by the Australian Tree Seed Centre (CSIRO, Australia) and germinated *in vitro* after one minute scarification in boiling water and sterilisation in 5% NaOCl for 15 min. Apical buds of plantlets one month old were used to initiate micropropagation.

In the case of *A. mangium*, three kinds of explants were used for susceptibility tests: micropropagated shoots obtained from (I) axillary buds of greenhouse plants, with 6 to 9 subcultures or (II) from *in vitro* germinated seeds, with 3 to 4 subcultures, and ((III) hypocotyls of seedlings one month old. For *A. mearnsii,* hypocotyls of one month seedlings or shoots of two months plantlets were inoculated.

***Agrobacterium strains***

Ten *A. tumefaciens* wild-type strains were tested. Nine of them are described in Table 1: A6 (Davis e Keathley, 1989), Ach5 (Sciaky et al., 1978), A281 (Guyon et al., 1980), B6 (De Greve et al., 1981), C58 (Casse et al., 1979), Chry5 (Bush e Pueppke, 1991), T37 (Sciaky et al., 1978), 82.139 (Michel et al., 1990). Strain A136, which does not induce tumour formation, was used as negative control of tumour induction. Strain Antib12 has not been characterized.

**Table 1**. *Agrobacterium tumefaciens* strains used in the study.

(Cepas de *Agrobacterium tumefaciens* testadas neste trabalho).

| Strain | Ti plasmid | Biovar | Opine* |
|---|---|---|---|
| A136 | | 1 | |
| A6 | pTiA6 | 1 | oct, agr |
| Ach5 | pTiAch5 | 1 | oct, agr |
| A281 | pTiBo542 | 1 | agr, man |
| B6 | pTiB6 | | oct, agr |
| C58 | pTiC58 | 1 | nop, agc |
| Chry5 | | 1 | chrys |
| T37 | pTiT37 | 1 | nop, agc |
| 82.139 | pTi82139 | 2 | nop, agc |

* agc - agrocinopine; agr - agropine; chrys - chrysopine; man - mannopine; nop - nopaline; oct - octopine

***Plant inoculation***

The stems were wounded with a scalpel previously dipped into the bacterial suspension, between the third and fourth node from the apex. Two concentrations were used, corresponding to optical density (O.D.) at 600 nm of 1 and 2. Hypocotyls were inoculated by wounding or hypocotyl pieces were immersed in the bacterial suspension. In some cases, acetosyringone (100 mM) was added to the bacterial suspension.

***Opine detection***

To detect opines in the tumors paper electrophoresis was used. For nopaline detection, the method of Otten e Schilperoort (1978) was used and for agropines, that of Reynaerts et al. (1988). The positive controls were tumors from tobacco plants inoculated with strains T37 and A281.

## RESULTS

**Acacia mangium**

Of the seven oncogenic strains tested, six induced tumour formation: A281, Antib 12, B6, Chry5, T37 and 82139 (Table 2).

The tumours appeared after 3 weeks and reached 4 to 5 mm of diameter after one month. Roots were formed from the stem at the wounding site or at the tumour base (Figure1). The results were very heterogeneous. They were related to the origin of plantlets (type I or II) and type of explant inoculated. When two bacterial concentrations were compared ($O.D._{600\ nm}$ 1 and 2), there was no difference between the percentages of tumour formation, except for strain Antib 12, where the tumour formation was

**Table 2**. Tumour formation induced by inoculation of *Acacia mangium* with *Agrobacterium tumefaciens* strains (for explant type, see text).

(Formação de tumores induzida por inoculação de *Acacia mangium* com cepas de *Agrobacterium tumefaciens* (tipo de explante: vide texto)).

| Strains | O.D.$_{600nm}$ | Explant type | Acetosyringone (100 mM) | Tumour formation (%*) |
|---|---|---|---|---|
| A136 | 1 | I shoot | - | 0 (64) |
| | 2 | I shoot | - | 0 (50) |
| | 1 | II shoot | - | 0 (54) |
| | 2 | II shoot hypocotyl | - | 0 (37) |
| | 1 | II shoot hypocotyl | - | 0 (74) |
| A281 | 1 | I shoot | + | 42.86 (70) |
| | 1 | I shoot | - | 48.59 (142) |
| | 2 | I shoot | - | 59.37 (32) |
| | 2 | II shoot | - | 20.00 (50) |
| | 2 | II shoot hypocotyl | + | 6.67 (30) |
| | 2 | II shoot hypocotyl | - | 0 (54) |
| Antib 12 | 1 | I shoot | - | 0 (33) |
| | 1 | II shoot | - | 0 (37) |
| | 2 | II shoot | - | 61.11 (18) |
| C58 | 2 | I shoot | - | 0 (32) |
| B6 | 1 | I shoot | + | 35.13 (37) |
| Chry5 | 1 | II shoot | - | 54.16 (24) |
| T37 | 1 | I shoot | + | 42.11 (19) |
| | 2 | I shoot | + | 50.00 (12) |
| | 2 | I shoot | - | 35.29 (17) |
| 82.139 | 2 | I shoot | + | 8.57 (35) |

* percentage of plantlets or hypocotyls forming tumor (total number of inoculated explants) - percentagem de plantas ou hipocótilos formando tumor (número total de plantas inoculadas)

higher at O.D. 2 than at O.D. 1. The presence of 100mM acetosyringone in the last bacterial suspension did not improve (A281), or improved very little the results (T37).

In case of strain A281, the highest percentages of tumour formation were obtained with type I plants, for an O.D. of 1 (48.59%) and 2 (59.37%). When inoculated with strain Antib12, type II plantlets formed tumours, but not those of type I. However, this last type of explants tumourized when infected with strains B6 and T37. Fragments of hypocotyls never formed tumour.

The tumours were separated from the plants and grown on hormone free MS medium. No differentiation was observed in the tumours.

Opines were not detected in the *Acacia* tumours, indicating a weak expression of bacterial genes, while agropine and nopaline were present in tumours from tobacco plants inoculated with strain A281 and T37 respectively.

***Acacia mearnsii***

Tumour formation was induced with strains A6, A281, Ach5, Antib12 and T37. The rate

varied between 8.33 and 61.11% according to the bacterial strain, the type of explant and inoculation (Table 3). The highest percentages were observed when the inoculation was made by wounding shoots with strains A6 and Ach5. With this species too, root formation occurred at tumour base or on the stem at the wouding site. When hypocotyls were infected, tumour formation was null or lower than 15%. Tumour differentiation was never observed.

**Figure 1**. Tumour and root formation on *Acacia mangium* shoot, four weeks after inoculation.

(Formação de tumores e raízes em caule de *Acacia mangium*, quatro semanas após a inoculação).

## DISCUSSION AND CONCLUSION

This work indicates that *Acacia mangium* and *A. mearnsii* plants are able to form tumours when inoculated with strains of *Agrobacterium tumefaciens. A. mangium* was responsive to tumour induction by several strains, except C58 and 82.139, but the experiments with these last strains were only carried with type I shoots (see Material and Methods). The origin and number of subcultures of the micropropagated plantlets,

**Table 3**. Tumour formation induced by inoculation of *Acacia mearnsii* with *Agrobacterium tumefaciens* strains (without acetosyringone).

(Formação de tumores induzida por inoculação de *Acacia mearnsii* com cepas de *Agrobacterium tumefaciens* (sem acetosiringona)).

| Wild-type strain | O.D.$_{600nm}$ | Explant type | Inoculation type | Tumour formation (%*) |
|---|---|---|---|---|
| A6 | 1 | shoot | wounding | 37.04 (27) |
| A136 | 1 | hypocotyl | wounding | 0 (36) |
| | 2 | hypocotyl | wounding | 0 (36) |
| A281 | 1 | hypocotyl | wounding | 8.33 (12) |
| | 1 | hypocotyl | immersion | 0 (36) |
| | 1 | hypocotyl | immersion | 0 (48) |
| Ach5 | 1 | shoot | wounding | 61.11(18) |
| Antib 12 | 1 | hypocotyl | wounding | 0 (32) |
| | 2 | hypocotyl | wounding | 14.81 (27) |
| | 2 | hypocotyl | immersion | 0 (12) |
| B6 | 1 | hypocotyl | wounding | 0 (32) |
| T37 | 1 | hypocotyl | wounding | 8.33 (24) |

* percentage of shoots or hypocotyls forming tumor (total number of inoculated explants) - percentagem de brotos ou hipocótilos apresentando tumor (número total de explantes inoculados)

as well as type of explant, are essential factors in this process.

The roots formed at the base of the tumour might regenerate due to a hormonal balance favourable to auxin in the inoculation site. The same strains, inoculated in *Eucalyptus* plantlets, induced the formation of shoot tumours (Machado et al., 1997).

For both species, when the inoculation of wild-type strains was made on excised explants, tumours never appeared, indicating that transformation occurs mainly on entire plantlets. This result is similar to that obtained by Limanton (1995) for *Acacia crassicarpa.* This could be due to differences between isolated explants and entire plantlets, as for example excretion of some substances from wounded tissues. It is well known that various substances, like sugars and phenolic compounds, can induce the virulence of *Agrobacterium* (Hooykaas e Beijersbergen, 1994). This could be the case of entire plantlets, that are more susceptible to *Agrobacterium tumefaciens* than the explants. However, the addition of acetosyringone, a phenolic compound, to the bacterial suspension, did not improve the results. This may be due to inappropriate concentration or application time of this compound. Differences of sensitivity to *Agrobacterium* were described for *Beta vulgaris* for which isolated explants were more susceptible to the bacteria inoculation than the plantlets (Godwin et al., 1992).

The results reported here will allow experiments of gene introduction into *Acacia* species tissues, provided that an efficient regeneration protocol be established.

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