Theor Appl Genet (1986) 72, 53–58



# Transformation of forage legumes using *Agrobacterium tumefaciens*

**K. J. Webb**

Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, Wales

Received July 23, 1985; Accepted October 25, 1985
Communicated by L. Alföldi

**Summary.** Galls were induced in six species of forage legumes following inoculation with wild-type strains of *A. tumefaciens.* The plant species was more influential than the bacterial strain in determining the type of tumour produced. Inoculation of *Medicago sativa* resulted in small, disorganised tumours. The three *Trifolium* species, *T. repens, T. hybridum* and *T. pratense,* formed galls which tended to produce roots and both *Onobrychis viciifolia* and *Lotus corniculatus* produced teratomatous galls. The shoots elongated in the latter species only. In *L. corniculatus,* tissues that were infected by five bacterial strains were capable of shoot regeneration when cultured on a hormone-free medium. The transformed nature of these shoots was confirmed by their failure to root, the production of callus from leaves cultured on hormone-free medium and the presence of opines.

**Key words:** *Agrobacterium tumefaciens* – Forage legumes – Crowngall – Transformation

## Introduction

*Agrobacterium tumefaciens* is a soil-borne bacterial pathogen which causes crowngall disease in many dicotyledonous species (De Cleene and De Ley 1976). During the infection process, a part (T-DNA) of the large bacterial plasmid (Ti plasmid) is transferred from the bacterium into the host's nuclear genome where it is transcribed. The expression of the T-DNA in the plant cell results in the growth of crowngall tumours, which continue to grow in the absence of exogenous hormones in culture, and in the production of the characteristic amino acids, the opines. The molecular genetics of crowngall disease has been comprehensively reviewed by Hooykaas and Schilperoort (1984).

This process of infection and transformation of cells by *A. tumefaciens* might be exploited for the incorporation of foreign DNA into crop plants. The Ti plasmids of this natural gene vector can be genetically modified to achieve the stable introduction and expression of foreign genes in plant cells (Bevan et al. 1983; Bevan 1984) and regenerated plants (Barton et al. 1983). Protoplasts of tobacco have been transformed by *A. tumefaciens* containing chimaeric genes constructed to confer resistance to antibiotics, such as kanomycin, on the plant cells. Plants that regenerated from these resistant cells have flowered and the foreign genes have been shown to be inherited in a stable way (De Block et al. 1984; Horsch et al. 1984).

The legumes are important crop species, some of which are known hosts of crowngall bacteria (see De Cleene and De Ley 1976). However, little progress has been made in the development of techniques for the transformation of the legumes. Soil-grown plants of *Glycine max* (Pedersen et al. 1983) produced galls at frequencies between 10–80% depending on the cultivar and the bacterial strain, while seedlings of *Medicago sativa* grown and inoculated in culture gave a maximum of 14% of infected plants (Mariotti et al. 1984). In neither of these species were teratomatous (shoot-forming) galls produced on the plants, nor were shoots regenerated from the transformed tissues in culture.

The objectives of this work were to test a range of forage legume species with several wild-type strains of *A. tumefaciens* in order to determine the ease of transformation and the characteristics of the tumours formed.

## Materials and methods

*Strains of A. tumefaciens*

Six wild-type strains of *A. tumefaciens,* A6, B6 and Ach5 (octopine strains) and T37, C58 and AT181 (nopaline strains) were obtained from the John Innes Institute, Norwich, UK. A cured strain (GV 3101), obtained from Max-Planck-Institute, Cologne, FRG, was used as a control in all experiments. The cultures were maintained on semi-solid medium ($TyCaCl_2$) containing 5 g/l Difco bacto tryptone, 3 g/l Difco yeast extract and 15 g/l Sigma agar (No. A7002). All cultures were incubated at 20 °C. Prior to the inoculation of all plant material, the bacteria were grown in agitated liquid $TyCaCl_2$ medium for 24 h at 25 °C. The virulence of the bacteria was tested using *Lycopersicon esculentum* cv 'Alicante' or stem segments of *Kalanchoe* sp.

*Inoculation of plants grown in soil*

Scarified seeds from six species and nine cultivars of forage legumes were germinated on moistened germination pads in petri dishes. After 7 days, the seedlings were transferred singly to 13 cm pots containing a soil-based compost with no added nitrogen. The plants were grown in a greenhouse maintained at a minimum temperature of 10 °C under the natural daylight of late spring to summer.

Each plant was inoculated twice – at 7 and 13 weeks of age – with one of the seven bacterial strains. The plant tissues were wounded and immediately inoculated using a hypodermic syringe and needle. Three plants (with 1–5 sites per plant) of each cultivar were inoculated with each bacterial strain. The sites inoculated depended on the species: stems and petioles in *L. corniculatus, M. sativa, T. hybridum* and *T. pratense;* stolons and petioles in *T. repens;* and shoot meristems and petioles in *O. viciifolia.* Each wounded area was wrapped in a piece of thermoplastic film (Nescofilm, Nippon Shoji Kaisha Ltd.) to maintain high humidity. After 21 days the film was removed and the sites marked with water-soluble acrylic paint.

At the second inoculation, the plants were cut back and, avoiding all previous inoculation sites, were treated as described above. The plants were watered through the bottom of the pot for three days after both the first and second treatments. The numbers of genotypes responding, the numbers of inoculation sites with galls and the approximate size and the general appearance of the galls were recorded when the plants were 25 weeks old (18 weeks from the first inoculation).

*Inoculation of plants grown in vitro*

Scarified seeds of *L. corniculatus* cv. 'Leo' were surface-sterilised for 30 min in 50% sodium hypochlorite (v/v) plus 1 drop of the wetting agent 'Tween', washed three times in sterile water, soaked for a further 30 min in 0.1% (w/v) $HgCl_2$ and washed six times in sterile water. The seeds were left to imbibe overnight, rinsed in sterile water and transferred onto paper wicks in 125 × 24 mm boling tubes containing 15 ml Jensen's medium (Jensen 1942) as modified by Rys (1982). The tubes were stoppered with permeable caps or cotton wool. After 28 days growth at 25 °C under a 24 h photoperiod, at least 10 seedlings were inoculated with each of the bacterial strains. The seedlings were inoculated by pinching the internodes at a specified number of sites (1–5) with forceps which had been dipped in the appropriate culture of bacteria. The data were recorded after 30 days from inoculation when the galls were excised and transferred to culture medium.

*Culture of crowngall tissue from plants grown in soil*

The galls were surface-sterilised in 50% sodium hypochlorite for 30 min, then washed three times in sterile water. The outer tissues were removed and the inner portions cultured on MS medium (Murashige and Skoog 1962, basal salts with 3% sucrose and no plant hormones). Proliferating cultures with no sign of bacterial or fungal contamination were transferred to fresh MS medium.

*Culture of crowngall tissue from plants of L. corniculatus grown in vitro*

The galls were excised, washed in 500 µg/ml carbenicillin and cut into small pieces and cultured on MS with 100 µg/ml carbenicillin. The proliferating cultures were maintained on this medium and subcultured every two weeks until they were free from bacteria.

Cultures inoculated with each of the five bacterial strains were selected. Twenty-five individual shoots were dissected out from each of the five culture lines and transferred to half-strength MS medium with 1% sucrose to encourage rooting. Several leaves were removed from each of these shoots and incubated on full-strength MS medium with 2% sucrose and no hormones. The presence or absence of callus production was noted after 14 days in culture.

All plant tissue cultures were incubated at 25 °C under continuous light from cool white fluorescent tubes at an intensity of 80 $\mu E/m^2/s$.

*Opine detection*

For the detection of nopaline, small pieces of tissue (2–3 mm in diameter) were incubated for 24 h at 25 °C in 20 µl MS medium with 10 mM L-arginine and 10 mM $\alpha$-ketoglutarate at pH 5.7 (Hernalsteens et al. 1984). This was modified for octopine detection by replacing 10 mM $\alpha$-ketoglutarate with 10 mM sodium pyruvate. The tissues were macerated with a plastic rod and centrifuged in a Bukard micro-centrifuge type-320a for 2 min. Samples of the supernatant (5 µl) were applied to Whatman 3 MM paper and dried. Electrophoresis was at 4 kV for 40 min in 5% formic acid, 15% acetic acid and 80% water. The papers were dried, dipped in a 1 : 1 solution 0.02% (w/v) phenanthrenequinone in 100% ethanol and 10% (w/v) NaOH in 60% ethanol (Otten and Schilperoort 1978). The dried electropherograms were viewed under u.v. light (366 nm). Pure synthetic nopaline and octopine were used as standards.

## Results

*Plants grown in soil*

The number of plants that produced tumours following inoculation with five of the wild-type strains of *A. tumefaciens* are shown in Table 1. The sixth strain, Ach5, and the control, GV3101, did not result in any gall formation in the legumes or the control plants *L. esculentum* and *Kalanchoe* sp. All of the species and cultivars of the forage legumes which were inoculated formed galls in response to at least two of the bacterial strains. Each plant was inoculated twice to determine whether the plant's response was consistent. Smaller galls were produced at a lower frequency at the second

**Table 1.** The proportion of sites and genotypes producting galls and the type of galls induced in a range of forage legumes inoculated with five strains of *Agrobacterium tumefaciens*

| Host plant | No. of plants with galls after inoculation with the following bacterial strains[a] | | | | | Plants forming galls | | % Inoculation sites forming galls in the responsive plants | | Ranges of gall diameter (mm) | Structures present on galls |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A6 | B6 | C58 | T37 | AT181 | Total | % | Inoculation 1 | Inoculation 2 | | |
| *T. repens* | | | | | | | | | | | |
| 'S. 100' | 2 | 2[b] | 3[b] | 3 | 1[b] | 11 | 73 | 70 | 78 | 1–10 | Roots |
| 'S. 184' | 3[b] | 3[b] | 3 | 3 | 1[b] | 13 | 87 | 83 | 55 | 1–10 | Roots |
| 'Donna' | 3 | 2[b] | 3 | 3 | 3[b] | 14 | 93 | 87 | 54 | 1–10 | Roots |
| *T. hybridium* | | | | | | | | | | | |
| 'Tetra' | 3[b] | 3[b] | 3 | 3 | 3[b] | 15 | 100 | 89 | 63 | 1–25 | Roots |
| 'Aurora' | 3 | 3 | 3 | 3[b] | 3[b] | 15 | 100 | 92 | 72 | 1–35 | Roots |
| *T. pratense* | | | | | | | | | | | |
| 'Norseman' | 3[b] | 0 | 3[b] | 3[b] | 3[b] | 12 | 80 | 43 | 50 | 1–15 | Roots |
| *O. viciifolia* | | | | | | | | | | | |
| 'Melrose' | 0 | 1 | 2[b] | 0 | 0 | 3 | 20 | 100 | 33 | 10–50 | Shoots |
| *M. sativa* | | | | | | | | | | | |
| 'Europe' | 3[b] | 0 | 2[b] | 3[b] | 2[b] | 10 | 67 | 55 | 19 | 1– 5 | None |
| *L. corniculatus* | | | | | | | | | | | |
| 'Leo' | 2[b] | 1 | 3[b] | 2[b] | 2[b] | 10 | 67 | 71 | 9 | 1–15 | Shoots |

[a] 3 plants from each cultivar were inoculated twice with each bacterial strain. The plants were approximately 7 and 13 weeks old at the 1st and 2nd inoculations respectively. The data were recorded when the plants were 25 weeks old

[b] In some of the plants, galls formed after either 1st or 2nd inoculation

attempt and some plants were inconsistent in their response. This was most marked in *T. pratense,* where six plants responded after the first inoculation and a different six after the second. However, the total number of susceptible plants from each cultivar (regardless of bacterial strain) was generally high. In most species 67–100% of the plants were successfully infected; the lowest infection rate was observed in *O. viciifolia* cv 'Melrose', in which only 20% of the plants formed galls.

The size and morphology of the galls varied considerably between species (Fig. 1). The galls of the *Trifolium* species (Figs. 1 A–C) varied from 1–35 mm in diameter, with *T. hybridum* generally producing the largest galls and *T. repens* the smallest. All three clover species produced some galls with roots. By contrast, the tumours of *M. sativa* were totally disorganised and consistently small (Fig. 1 D). Both *O. viciifolia* and *L. corniculatus* formed teratomatous galls. The galls that formed on the petiole of *O. viciifolia* were disorganised (Fig. 1 E), while the exceptionally large galls that were produced at the shoot meristem showed occasional shoot-like structures which never developed further. These galls generally prevented any further growth of the plant. Only in *L. corniculatus* were teratomatous galls produced that were capable of shoot elongation (Fig. 1 F), and then only in tissues transformed with bacterial strains AT181, C58 and T37.

Tumours from all six species were cultured on MS medium and disorganised callus cultures were produced in five of them. In the sixth species, *L. corniculatus,* cultures which regularly regenerated shoots were produced from tissues inoculated with strains B6, A6, AT181, C58 and T37.

*L. corniculatus cv. 'Leo' seedlings grown in vitro*

The data in Table 2 show that between 10–80% of the seedlings formed galls (Fig. 2 A) depending on the bacterial strain used. Many of the remaining seedlings showed swellings at the infection site.

In total, six wild-type bacterial strains were used to transform these *L. corniculatus* seedlings. All strains produced galls and swellings, with Ach5 being the least effective gall-producer. Strain AT181 produced either teratomatous or disorganised galls, while A6, T37 and C58 also induced root formation. Strain B6 never produced teratomas, only disorganised galls with occasional roots. All of these galls were cultured on MS medium. Tissues inoculated with strain Ach5 failed to grow, but galls from about 50% of the remaining plants survived in culture to regenerate shoots. Most of these shoots were abnormal, with small leaves, thickened stems and many branches, and failed to produce roots. The ability of these shoots to elongate varied with the bacterial strain used to inoculate the tissues (Fig. 2 B).

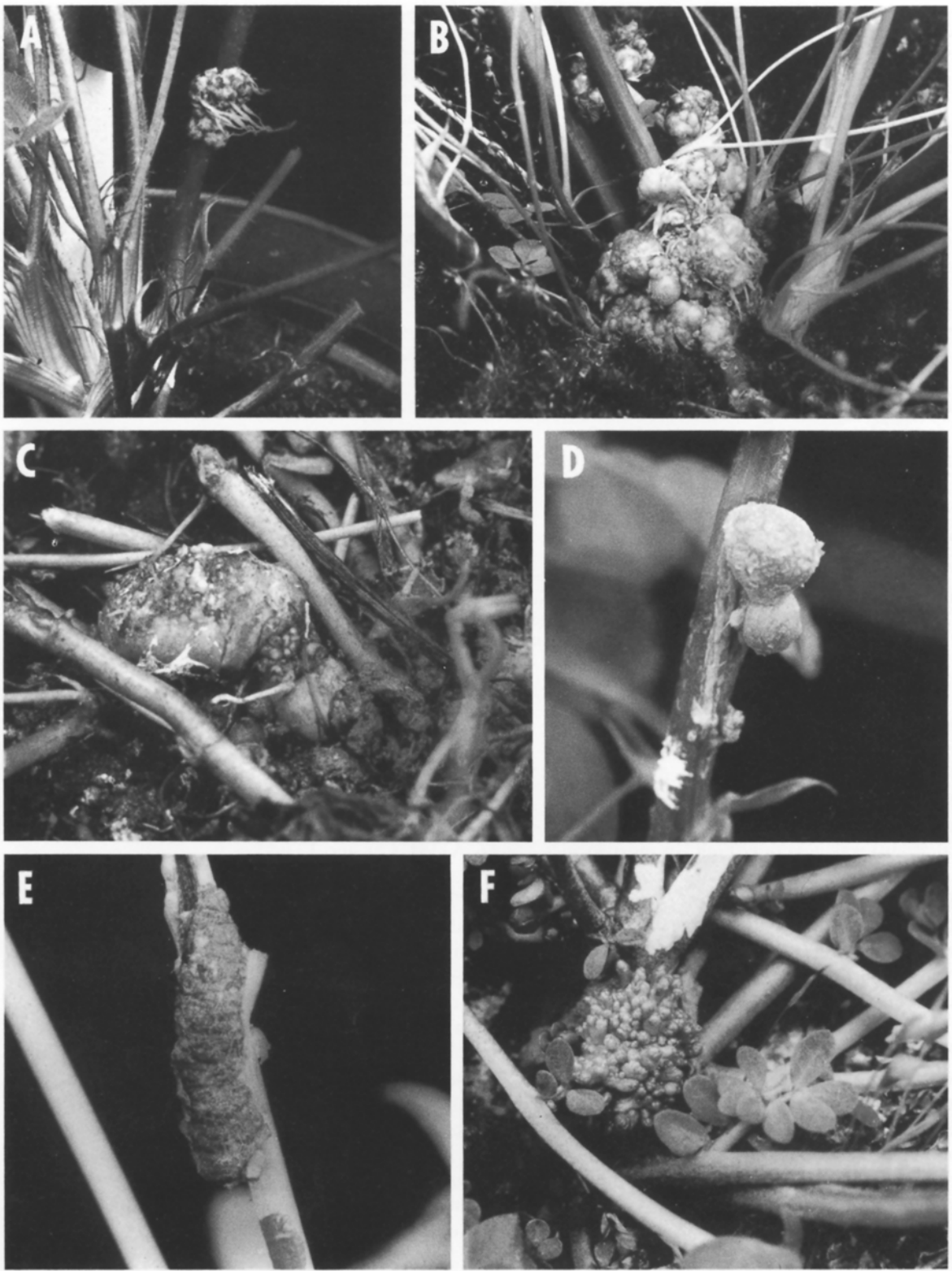


**Fig. 1 A–F.** Galls formed on greenhouse-grown plants, 18 weeks after inoculation with *A. tumefaciens*. The plants in **A, B, D, E** and **F** were infected with strain C58, the plant in **C** was infected with strain T37. **A** *T. pratense* cv 'Norseman' (×1.0); **B** *T. hybridum* cv 'Aurora' (×0.8); **C** *T. repens* cv 'S. 100' (×1.8); **D** *M. sativa* cv 'Europe' (×4.0); **E** *O. viciifolia* cv 'Melrose' (×4.0); **F** *L. corniculatus* cv 'Leo' (×4.0)

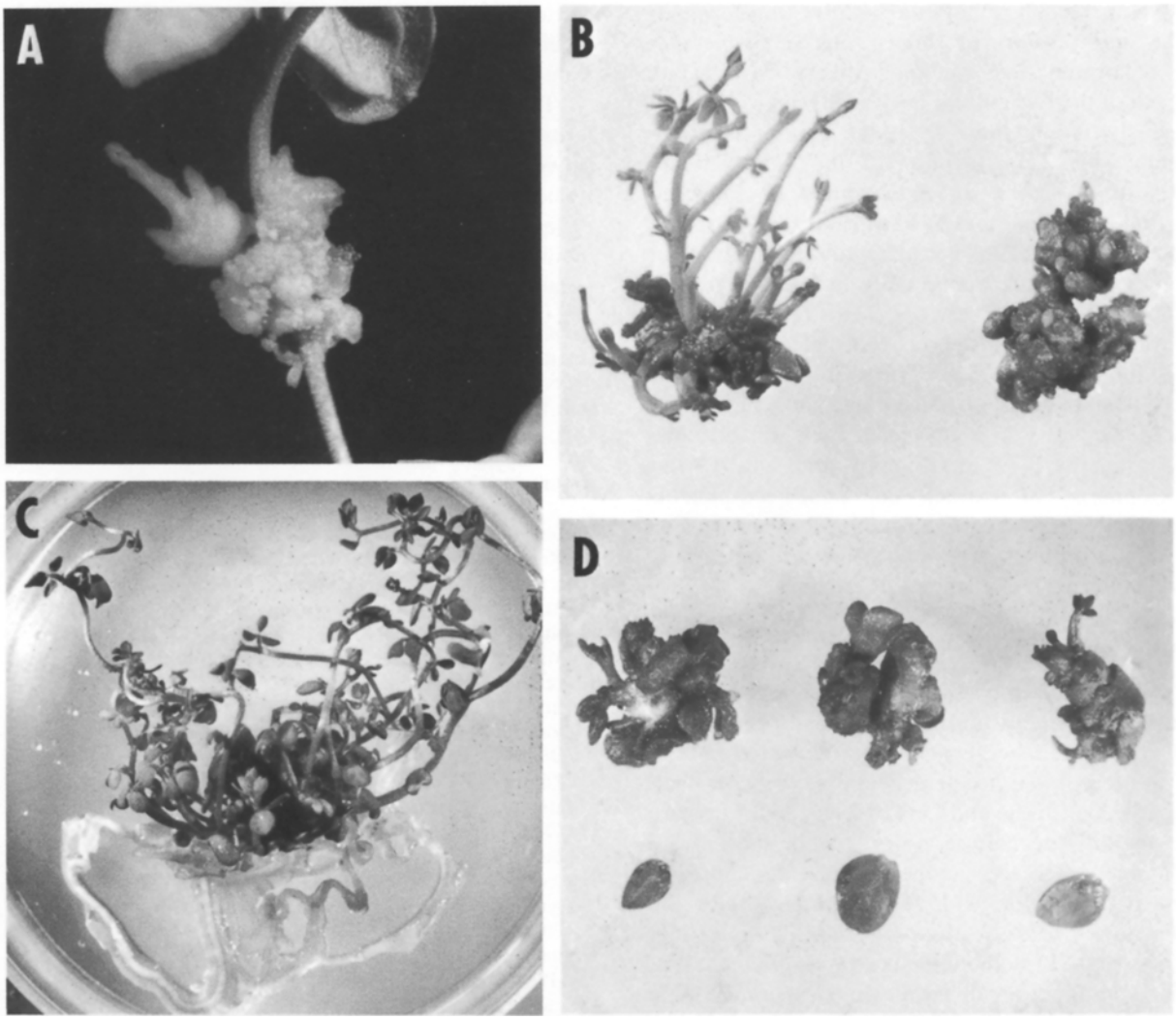


**Fig. 2A–D.** Tissues of *L. corniculatus* cv. 'Leo' grown in vitro. **A** Gall produced on stem segment of seedling inoculated with strain C58 (×6); **B** Transformed tissue induced by strains B6 (*left*) and AT181 (*right*) cultured on MS medium. Note the stunted shoots in tissues transformed by strain AT181 and the absence of roots in both (×1.5); **C** Normal shoot regenerated from cotyledons incubated on MS with 0.5 mg/l NAA and 0.5 mg/l BAP (×1); **D** Leaves from shoots transformed with strain A6 (*above*) and normal shoots (*below*) after 24 days incubation on MS medium. Transformed leaves have proliferated, producing callus and shoots (×2.5)

**Table 2.** Gall formation on seedlings of *Lotus corniculatus* cv 'Leo' grown in vitro

| Bacterial strain | No. of plants inoculated | No. of sites inoculated | % plants with galls[a] | % sites with galls[a] | Average size of galls (mm) | Appearance of galls | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | On seedling[b] | | | In culture |
| | | | | | | D | S | R | |
| A6 | 12 | 35 | 67 (92) | 43 (71) | 2.7 | + | + | + | Shoots |
| B6 | 11 | 33 | 45 (100) | 18 (79) | 1.8 | + | – | + | Shoots |
| Ach5 | 10 | 26 | 10 (100) | 4 (85) | 1.0 | – | – | + | No growth |
| C58 | 10 | 25 | 80 (90) | 72 (84) | 2.5 | + | + | + | Shoots |
| T37 | 10 | 31 | 80 (100) | 55 (87) | 2.5 | + | + | + | Shoots |
| AT181 | 10 | 28 | 20 (90) | 11 (75) | 3.0 | + | + | – | Shoots |
| GV 3101 (control) | 11 | 33 | 0 (0) | 0 (0) | 0 | – | – | – | No growth |

[a] Figures in brackets include plants with swellings at the infection sites not classified as galls
[b] D: disorganised; S: shoots; R: roots; +: presence; –: absence

Leaves excised from both the putative transformants and the non-transformed shoots (Fig. 2C) were cultured on hormone-free medium. After 14 days, leaves from each of the five culture lines produced callus (and later shoots), while those from the control failed to proliferate (Fig. 2D). In four of the culture lines, 80–100% of the shoots were transformed, as evidenced by the ability of the leaves to form callus and shoots in the absence of exogenous hormones and the inability of the shoots to produce roots. Only in cultures treated with strain AT181 were very few shoots (ca 25%) found to be transformed. No opines were detected in these cultures infected by strain AT181. This is presumably related to the low level of transformed shoots that were produced. Opines were detected in the cultures transformed by stains T37 and C58 (nopaline) and A6 and B6 (octopine). The extracts contained components which migrated to the same position as the appropriate opine standard on electrophoresis, confirming the expression of the T-DNA in these culture lines.

## Discussion

Galls were formed in all species of the forage legumes chosen for this study. Three of the six species used, namely *T. repens, T. pratense* and *M. sativa,* have previously been reported to be susceptible to crowngall disease (see De Cleene and De Ley 1976; Mariotti et al. 1984). In the greenhouse-grown plants there were considerable differences in the responses of these six species to inoculation with *A. tumefaciens.* With the exception of *O. viciifolia,* the proportion of susceptible plants was high. In both cultivars of *T. hybridum,* all of the plants produced galls while in *O. viciifolia,* only 20% were responsive. The differences in growth habits of the plants – and consequently the type of tissues that were inoculated – may be important in determining their responses.

A strong effect of genotype has previously been reported by Mariotti et al. (1984) on gall formation in seedlings of *M. sativa.* In their experiments, five cultivars were inoculated by decapitating the seedlings and inoculating the wounded surface. The best responses were observed using strain Ach5, which induced 14% of plants of cv 'Sabilt' and 12% of plants of cv 'Europe' to form galls after 30–35 days. In the greenhouse experiments described here, 67% of plants of cv Europe formed galls. The difference may reflect the time allowed for galls to develop or the different conditions employed. The decapitation of *L. corniculatus* seedlings followed by placing a drop of the bacterial suspension culture on the cut surface resulted in about 8% of the seedlings forming galls (unpublished results).

In the experiments described here, similarly high rates of infection were produced both by inoculating plants of *L. corniculatus* grown in the greenhouse with a syringe needle and by inoculating seedlings grown in vitro with forceps coated in bacteria. The galls produced by the latter system were easy to excise and culture. These galls proliferated on the hormone-free medium and readily regenerated large numbers of transformed shoots.

The ease with which *L. corniculatus* can be transformed by wild-type strains of *A. tumefaciens* and its proven ability to regenerate shoots readily from normal tissues (Webb et al. 1985) allows the possibility of developing this system with a view to using genetically modified non-tumorigenic strains to achieve the incorporation and expression of foreign genes in this species.

*Acknowledgements.* I wish to thank Ian Armstead for his assistance in testing for the presence of the opines, Dr. Chris Pollock for his guidance on paper electrophoresis and Dr. Phil Dale for his critical reading of the manuscript.

## References

Barton KA, Binns AN, Matzke AJM, Chilton M-D (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033–1043

Bevan MW, Flavell RB, Chilton M-D (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187

Bevan MW (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681–1689

De Cleene M, De Ley J (1976) The host range of crown gall. Bot Rev 42:389–466

Jensen HL (1942) Nitrogen fixation in leguminous plants. 2. Is symbiotic nitrogen fixation influenced by *Azotobacter?* Proc Linn Soc NSW 67:205–212

Hernalsteens J-P, Thia-Toong L, Schell J, Van Montagu M (1984) An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis.* EMBO J 3:3039–3041

Hooykaas PJJ, Schilperoort RA (1984) The molecular genetics of crown gall tumorigenesis. In: Scandalios JG, Caspari EW (eds) Adv Genet, vol 22. Molecular genetics of plants, pp 209–283

Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, Hoffmann N (1984) Inheritance of functional foreign genes in plants. Science 223:496–498

Mariotti D, Davey MR, Draper J, Freeman JP, Cocking EC (1984) Crown gall tumorigenesis in the forage legume *Medicago sativa* L. Plant Cell Physiol 25:473–482

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Otten LABM, Schilperoort RA (1978) A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim Biophys Acta 527:497–500

Pedersen HC, Christiansen J, Wyndaele R (1983) Induction and in vitro culture of soybean crown gall tumours. Plant Cell Rep 2:201–204

Rys GJ (1982) Aspects of nitrogen fixation by white clover (*Trifolium repens* L.). PhD Thesis, University College of Wales, Aberystwyth

Webb KJ, Woodcock S, Pike LS, Dale PJ (1986) Plant regeneration in the forage legumes. In: Withers LA, Alderson PG (eds) Plant tissue culture and its agricultural applications. Butterworth Scientific, London (in press)