

## Condensed tannin levels in different tissues and different developmental stages of transformed and non-transformed Lotus corniculatus.

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### Introduction

Condensed tannins are polymers of flavan-3,4-diols joined by 4-8 interflavan bonds with a flavan-3-ol at the 4' terminal end, and these compounds are accumulated by many plants, including many *Lotus spp.* The condensed tannin of *Lotus corniculatus* is comprised of the flavan-3,4-diols leucocyanidin and leucodelphinidin, which release cyanidin and delphinidin upon acid hydrolysis.

*L.corniculatus* accumulates condensed tannins in its roots, leaves, shoots and flowers. The levels of condensed tannin varies between these tissues, and between different genotypes, and may well be under developmental control, as is the case in other species (Bell *et al* 1992). *Agrobacterium rhizogenes* transformed *L.corniculatus* is currently being used as a model system for the study of, and genetic manipulation of, condensed tannin biosynthesis (Morris and Robbins 1992, Robbins *et al* 1992, Carron *et al* 1993) and a number of genotypes of *L.corniculatus* have been selected and clonally micropropagated for transformation.

This study documents the detailed analysis of the tannin accumulation in the green tissue of a transformed and a non-transformed plant of one of the selected genotypes, s50.

### Experimental

**Growth of plants:** Transformed and non-transformed plants were grown in a transgenic growth room at 20°C with a 16 hr day.

**Determination of condensed tannin levels in green tissue:** To remove the chlorophyll from the green tissue, between 200 and 400mg of tissue was placed into a 15ml screw cap tube with 5 mls of 70% ethanol and incubated at 80°C for 10 minutes. The 70% ethanol was decanted off, and replaced with 5mls of 100% ethanol and incubated for a further 10 minutes at 80°C. The 100% ethanol was decanted off and replaced with a further 5mls of 100% ethanol and incubated for a further 10 minutes. The ethanol was decanted off the tissue, and the ethanol extracts were pooled.

The tannins were hydrolysed to anthocyanidins by incubating the decolorised tissue with 2mls of butanol:HCl (95:5) at 100°C for 1hr. The butanol:HCl was allowed to cool, and the absorbance spectrum determined between 400-700nm. The absorbance due to anthocyanidins at 550nm was determined by interpolating the underlying curve, not part of the 550nm peak, and subtracting the absorbance of this at 550nm from the total absorbance at 550nm. This figure was used to calculate the tannin levels using an E<sup>1%</sup> value of 150 (Stafford and Cheng 1980).

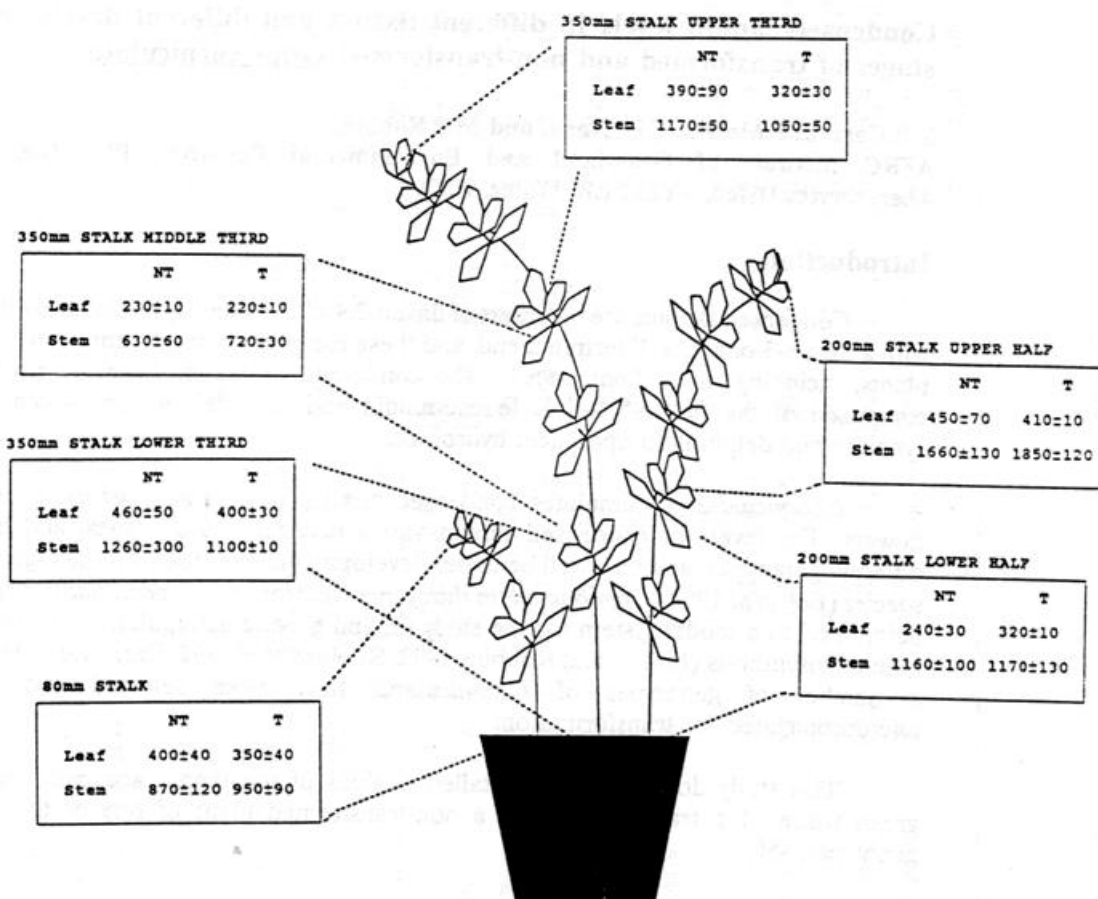


Figure 1. Schematic diagramme of a *L. corniculatus* plant, genotype *s50*, showing the distribution of condensed tannins in different tissues. Boxed tables show the levels in leaves and stems of transformed (T), and non-transformed (NT) plants. Tannin levels are given in  $\mu\text{g Tannin/g}$  fresh weight tissue.

To estimate the levels in pooled extracts, the ethanol was driven off by heating to 40°C with a compressed air flow in a Techne SC3 sample concentrator, until only 1ml of sample remained. This was made up to 5mls with distilled water, and extracted three times with an equal volume of hexane. The aqueous phase was reduced to dryness at 40°C under air flow. The residue was redissolved in 2mls of Butanol:HCl and the tannin determined as above.

Tannin determinations from single leaves was performed as above except that 1ml aliquots of 70% and 100% ethanol were used, and the hydrolysis was carried out in 1ml of butanol:HCl.

The percentage procyanidin of the tannin was determined as described by Morris and Robbins (1992).

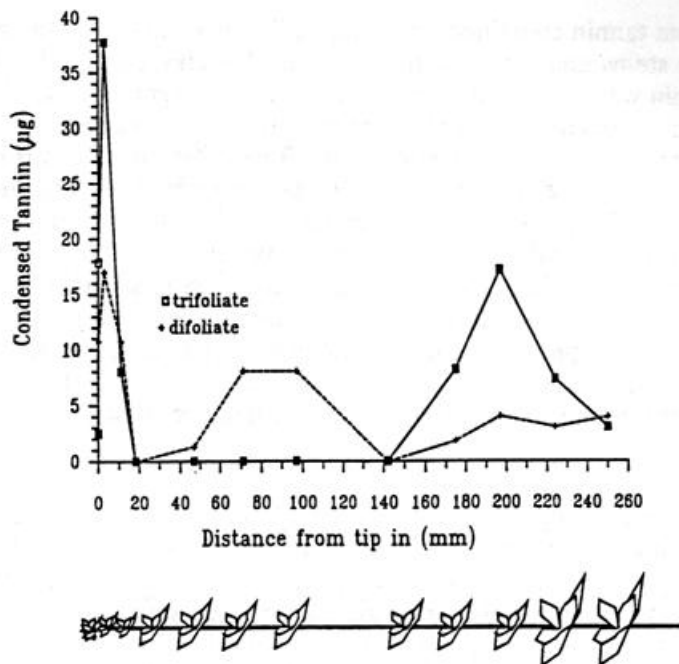


Figure 2. Graph showing the amount of condensed tannin in individual bifoliate and trifoliate leaves of a nontransformed plant of *L.corniculatus*.

## Results

**Tannin distributions in transformed and non transformed plants:** Two plants of the same genotype (s50), one transformed with *Agrobacterium rhizogenes*, and one non transformed, were analysed. The stalks of the plants were cut from the roots and sorted into seven size categories based on the length of the stalk. These were, >60mm, 60-100mm, 100-160mm, 160-240mm, 240-320mm, 320-380mm, and >380mm. Three of these size categories, 60-100mm (80mm), 160-240mm (200mm), and 320-380mm (350mm) were subdivided. The 80mm category was divided into stem and leaf; the 200mm category into upper half and lower half, and then into stem and leaf; and the 350mm category into upper third, middle third, and lower third, and then into stem and leaf. Triplicate tannin determinations were performed on all these categories.

None of the ethanol extracts of these plants gave higher than background optical density at 550nm after butanol hydrolysis, indicating that they did not contain ethanol soluble condensed tannin. However, other genotypes of *L.corniculatus* have been shown to contain ethanol soluble condensed tannins (data not shown). No significant difference was found between the tannin levels in the transformed and non transformed plants in any of the categories measured. However large differences were found between the different classes.

The greatest difference in levels was found between leaves and stems. Leaves contained on average 30% of the tannin contained in stem tissue, and also had a very different procyanidin content. Leaf tannin on average consisted of 68% procyanidin,

where as stem tannin contained on average 27% procyanidin. Tannin levels also varied between the stems and leaves of different length stalks, though the ratio of leaf tannin to stem tannin was consistent across the different categories (Fig. 1).

Tannin levels in individual leaves of a stem of *Lotus corniculatus*: Tannin analysis was performed on individual leaves of a 250mm stem of a non-transformed plant of genotype s50, bifoliate and trifoliate leaves were sampled separately. The analysis showed that tannin levels varied greatly depending on the position of the leaf. Highest tannin levels were found in the very young leaves closest to the top of the stalk. Leaves positioned at the middle of the stalk contained much less tannin than those at the top, the tannin levels being undetectable in trifoliate leaves positioned between 18mm and 142mm. The leaves positioned at the base of the stalk contained greater levels of tannin than those at the middle though less than those at the very top (Fig. 2). This distribution was also confirmed by vanillin/HCl staining (data not shown).

## Discussion and Conclusions

The observation that the condensed tannin levels in transformed and non transformed plants are comparable suggests that transgenic *L. corniculatus* is a suitable system for the study of, and genetic manipulation of, condensed tannin biosynthesis. However as the tannin levels varied greatly between stem and leaf, and between stems and leaves at different stages of development it is important to ensure that any comparisons made are between equivalent developmental stages, or between representative averages of all developmental stages. The procyanidin content of leaf tannins was very different from that of stem tannins. This indicates that the tannins of leaves and stems have different compositions, and suggests that the separate sampling of leaves and stems is essential for the production of interpretable comparisons.

The condensed tannins in individual leaves of a single stalk also varied greatly. The absolute tannin levels were much higher in the young leaves close to the top of the stalk than in older leaves toward the middle of the stalk, and this suggests that as a leaf ages the absolute tannin level drops. This infers that the tannins are either subject to catabolic turnover or are converted to compounds which neither releases proanthocyanidins upon acid hydrolysis, nor stain red with vanillin/HCl.

## References

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