# Alkaloids in Stem Roots of *Nicotiana tabacum* and *Spartium junceum* Transformed by *Agrobacterium rhizogenes*

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The growth of root hairs from stems of *Nicotiana tabacum* and *Spartium junceum* was induced by *Agrobacterium rhizogenes*. Stem roots of *Nicotiana* contained nicotine and a second alkaloid, probably anabaseine. Root hairs from stems of *Spartium* contained cytisine, N methylcytisine and anagyrine as major alkaloids. The alkaloid content of the tumors was 3–12 times higher than that of the respective healthy host tissue.

*Agrobacterium tumefaciens* and *A. rhizogenes* contain plasmid encoded genes which allow them to transform a host plant to generate proliferating "tumors". These plasmids, *i.e.* the Ti- or Ri-plasmid respectively, provide interesting vectors for the genetic engineering of higher plants [1–2]. The typical tumor tissue of *A. tumefaciens* consists of knob-like structures, while *A. rhizogenes*-induced tumors consist of long root-like outgrowths (Fig. 1).

It has previously been shown that the metabolism of the transformed tissue changes. First of all the infected cells produce opines, which the bacteria can use as a nitrogen source [3]. But also the secondary metabolism seems to be influenced. Flores and Filner [4] found that roots obtained from Solanaceous plants after infection with *Agrobacterium rhizogenes* produced significantly more alkaloids than normal tissues. We have studied whether this observation holds true for other plant species and report in this communication on the alkaloid metabolism in root hairs of stems from *Nicotiana tabacum* and *Spartium junceum*, species which produce pyridine or quinolizidine alkaloids respectively.

*Abbreviations:* GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography – mass spectrometry; FW, fresh weight; FID, flame ionization detector; PND, nitrogen specific detector; RI, Kovats retention index.

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## Material and Methods

### *Plants*

During the winter months plants of *Spartium junceum* and *Nicotiana tabacum* were grown in a green house at 20 °C and c. 70% relative humidity, during summer months in the experimental garden under natural conditions.

### *Agrobacterium rhizogenes*

The *Agrobacterium* was grown on AB-medium as described in [4]. Five to 14 day-old cultures were used for the infection experiments. Incisions of 3–4 cm length were made into the bark and cambial tissue of stems usually of flowering plants. About 20 µl bacterial mass was smeared into the wound with a flat spatulum. Afterwards the wounds were tightly wrapped with 3–4 layers of parafilm. When the infection was successful, we could observe the outgrowth of root hairs (Fig. 1), which could reach a length of up to 8 cm.

### *Alkaloid analysis*

Plant material was homogenized in 0.5 M HCl and left standing for 30 min at room temperature. After separation of the cellular debris by centrifugation, the supernatant was made alkaline with 6 M NaOH. About 20 ml of the homogenate was applied onto a standard extrelut column (Merck, Darmstadt). The alkaloids were eluted with $Cl_2CH_2$. Alkaloid extracts

were analyzed by capillary gas-liquid chromatography as described in [5–7] using a Perkin Elmer gas chromatograph (Sigma 1b) equipped with both flame ionization and nitrogen specific detectors. A DB-5 capillary column (30 m × 0.3 mm) was employed.

*Identification of alkaloids*

Capillary GLC is a powerful tool for the analysis of complex alkaloid mixtures and is routinely used in our laboratories. Alkaloids were identified by their specific Kovats retention indices, determined in previous experiments [5–8]. To confirm this identification all extracts were analyzed by capillary GLC-mass spectrometry, which allows an unambiguous identification in most instances.

*Nicotiana tabacum:* Nicotine: RI = 1307; $M^+$ = 162 (15%), 133 (17), 84 (100), 42 (20); anabasine: RI = 1435; $M^+$ = 162 (15), 133 (20), 119 (20), 105 (45), 84 (100); an alkaloid which probably is anabaseine: RI = 1457; $M^+$ = 160 (60), 131 (20), 117 (10), 105 (40), 80 (30), 54 (100).

*Spartium junceum:* N-methylcytisine: RI = 1955; $M^+$ = 204 (20), 160 (10), 146 (5), 58 (100); cytisine: RI = 1995; $M^+$ = 190 (90), 160 (109), 147 (100), 146 (30), 134 (10); 5,6-dehydrolupanine: RI = 2130; $M^+$ = 246 (50), 136 (5), 98 (100), 97 (50); lupanine: RI = 2170; $M^+$ = 248 (38), 219 (6), 149 (62), 136 (100), 110 (20); anagyrine: RI = 2373; $M^+$ = 244 (30), 160 (59), 146 (10), 136 (15), 98 (100).

Fig. 1. Stem tumors (root hairs) appearing from a stem of *Nicotiana tabacum* infected with *Agrobacterium rhizogenes*.

## Results and Discussion

*Host range of Agrobacterium rhizogenes*

*Agrobacterium rhizogenes* can infect a wide variety of dicotyledenous plant species including many species of Solanaceae. Leguminosae seem to be less susceptible to the *Agrobacterium* infection. In our experiments, nearly 100% of the infections were successful in *Nicotiana* plants. Root hairs were observed to protrude out of the wounds after 2 weeks, which grew vigorously (Fig. 1).

With quinolizidine alkaloid producing species the situation was different. No infection was achieved in *Cytisus scoparius, Laburnum anagyroides, Thermopsis fabacea, Lupinus arboreus, L. albus* (both in alkaloid-rich and "sweet" varieties) and *Lupinus polyphyllus* although the infection was repeated several times varying age and amount of the inoculum. About 10% of all infections were positive in *L. mutabilis*. Here the tumors stopped to grow when they pierced the parafilm. More successful were the experiments with *Spartium junceum,* in which more than 50% of the infections became positive. If the wounds remained closely sealed by parafilm, long root hairs were formed, otherwise knoblike tumors appeared.

When stems with *Agrobacterium* induced roots were cut and put into tap water, we observed a rapid growth of the roots. Non-induced stems formed no roots *(Spartium)* or few roots under these conditions *(Nicotiana)*. We brought the stems into normal garden soil, when plenty of roots had been formed. This procedure resulted in healthy growing plants. Thus *Agrobacterium rhizogenes* might help to multiply plants which are otherwise difficult to propagate.

*Alkaloid metabolism*

We excised tumor tissue from the host tissue, extracted the alkaloids and determined the alkaloid patterns and alkaloid concentrations by capillary GLC and GLC-MS. As controls we chose stem parts which were 10 cm apart from the infected site or from uninfected stems (Tables I, II).

The alkaloid pattern of *Spartium* tumors was similar to that of the healthy tissue, but N-methylcytisine was the major alkaloid whereas it is cytisine in the non-infected tissues (Fig. 2). The alkaloid content of tumor tissue was about 2–3 times higher than that of the respective control (Table I). Since QA are formed in the leaves or in the case of *Spartium* also in the chlorophyllous stems, this result is somewhat surprising as *de novo* synthesis in the root tumors (which contain no chlorophyll) seems to be unlikely. We have shown previously that lupins respond to wounding in boosting the alkaloid content by a factor of

Table I. Alkaloid composition of *Spartium junceum* analyzed by capillary GLC (Fig. 2). A. Alkaloids of healthy shoots. B. Alkaloids of stem roots, induced by *Agrobacterium rhizogenes*. Alkaloids: 1, N-methylcytisine; 2, cytisine; 3, 5,6-dehydrolupanine; 4, lupanine; 5, anagyrine; +, trace.

| Plant | Alkaloids [µg/g FW] | | | | | Total alkaloids [µg/g FW] |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| 1 A | 65 | 188 | 16 | 8 | 48 | 325 |
| 1 B | 687 | 157 | 14 | 14 | 114 | 986 |
| 2 A | 27 | 82 | 14 | 14 | 41 | 178 |
| 2 B | 99 | 99 | 11 | + | 33 | 242 |
| 3 A | 63 | + | 10 | + | 30 | 103 |
| 3 B | 241 | 40 | 13 | 13 | 80 | 387 |

Table II. Alkaloids of *Nicotiana tabacum* separated by capillary GLC (Fig. 2). A. Alkaloids of stem roots, induced by *Agrobacterium rhizogenes*. B. Alkaloids of healthy stem tissue. Alkaloids: 1, nicotine; 2, anabaseine (?).

| Plant | Alkaloids [µg/g FW] | | Total alkaloids [µg/g FW] |
|---|---|---|---|
| | 1 | 2 | |
| 1 A | 79 | 68 | 147 |
| 1 B | 65 | – | 65 |
| 2 A | 195 | 42 | 237 |
| 2 B | 60 | – | 60 |
| 3 A | 658 | 167 | 825 |
| 3 B | 115 | – | 115 |
| 4 A | 845 | 61 | 906 |
| 4 B | 65 | – | 65 |
| 5 A | 1532 | 152 | 1684 |
| 5 B | 85 | – | 85 |

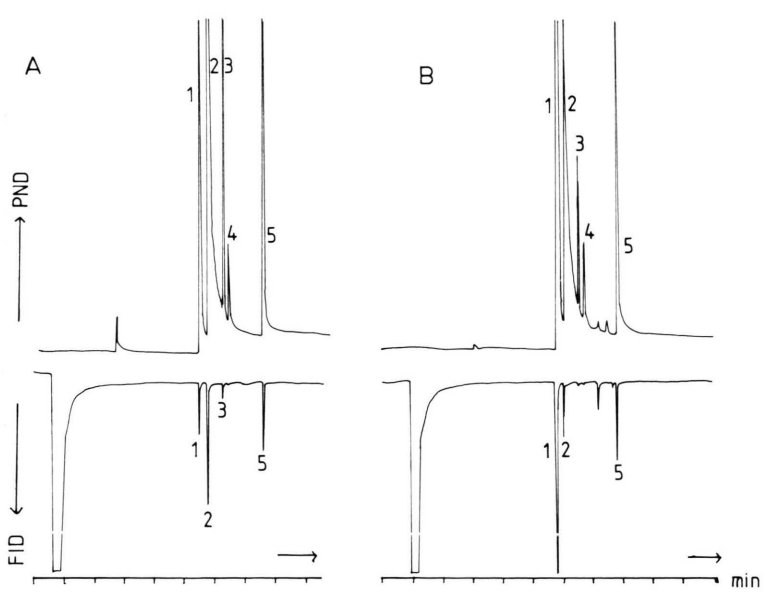


Fig. 2. Separation of alkaloids from *Spartium junceum* by capillary GLC and detection by flame ionization and nitrogen specific detectors. A. Alkaloids from healthy stems. B. Alkaloids from root hairs of *Spartium* stems after infection with *A. rhizogenes*. Alkaloids: 1, N-methylcytisine; 2, cytisine; 3, 5,6-dehydrolupanine; 4, lupanine; 5, anagyrine.

2–4 within a few hours [9]. Whether the wound response plays a role in the phenomenon observed here has to be determined yet.

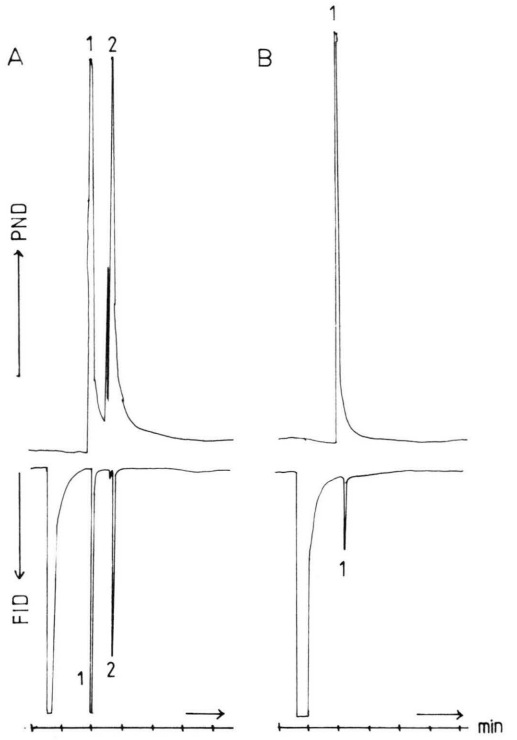


Fig. 3. Separation of alkaloids from *Nicotiana tabacum*. A. Alkaloids from root hairs of *Nicotiana* stems transformed with *A. rhizogenes*. B. Alkaloids of healthy stems. Alkaloids: 1, nicotine; 2, probably anabaseine.

Nicotine, which was found in root hairs of *Nicotiana* stems, accounted for 80–90% of total alkaloids. Minor alkaloids were anabaseine and a third alkaloid which probably was anabasine (Fig. 3, Table II). Healthy stem tissue did not show these minor alkaloids. In *Nicotiana* the alkaloids are usually formed in the root. Thus the more diverse alkaloid pattern found in transformed root tissue might be an indication that active alkaloid synthesis was going on in the root hairs. The alkaloid content was up to 12 times higher in the root tumors than in the healthy stem tissues which gives further evidence of active alkaloid metabolism in transformed stem tissue which behaves as root tissue.

Both examples show, that both the alkaloid content and the alkaloid pattern are changed significantly in tumor tissues. It has already been demonstrated that root cultures obtained from *Hyoscyamus* tumors were superior in terms of growth and alkaloid production as compared to normal root cultures [4]. Therefore, *Agrobacterium rhizogenes* offers an interesting approach to manipulate the production of secondary metabolites, especially of those products which are synthesized by root tissue.

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