

# Induction of Pyridine Alkaloid Formation in Transformed Root Cultures of *Nicotiana tabacum*

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Hairy root cultures of *Nicotiana tabacum* were set up by excised root tips with *Agrobacterium rhizogenes*. The successful transformation was confirmed by analyzing *rolC* and *virC* genes using polymerase chain reaction (PCR). Hairy root cultures were employed to study the formation of pyridine alkaloids, mainly nicotine. The transformed cultures were incubated with potential elicitors, such as methyljasmonate, quercetin and salicylic acid, in order to stimulate the biosynthesis of pyridine alkaloids. Profile and amounts of pyridine alkaloids were analyzed using capillary GLC-MS. Treatment of the cultures with methyljasmonate (50  $\mu$ M) increased the alkaloid accumulation ca. 7-fold up to a level of 0.58 mg/g fresh weight as compared to untreated controls. Quercetin (200  $\mu$ M) enhanced the alkaloid production ca. 4-fold (0.34 mg/g fresh weight) within 24 h. In contrast salicylic acid in all tested concentrations decreased the alkaloid level to 1  $\mu$ g/g fresh weight. Also the inhibitory effect of salicylic acid on the elicitation effect of methyljasmonate and quercetin was investigated.

**Key words:** *Nicotiana tabacum*, Pyridine Alkaloids, Hairy Root Culture

## Introduction

Tobacco, *Nicotiana tabacum*, is a pyridine alkaloids-bearing plant. Alkaloids, represented by nicotine as the major secondary metabolite, are agonists at the nicotinic acetylcholine receptor and thus are psychoactive and neurotoxic agents. There are also minor tobacco alkaloids, e.g. nor-nicotine, anabasine, and anatabine, but they are relatively less potent compared to *S*-(-)-nicotine. Nicotine is the predominant alkaloid in a number of *Nicotiana* species and may function as a defensive toxin by acting on the nervous system of herbivores (Wink, 1988, 2007).

Most alkaloids are important for the fitness and survival of the plants producing them, since they help to protect against herbivores and/or infect microorganisms (Harborne, 1993; Wink, 1988, 1999a, b, 2008). The alkaloid formation is not static but under the regulation of internal and external factors. Upon attack or infections, secondary metabolism is enhanced in many plants; either the biosynthesis of new compounds (phytoalexins) takes place or the concentration of already existing compounds is increased (Wink, 1999a, b).

Jasmonic acid and its ester methyljasmonate have been found to be elements of a signal pathway leading to the induction of secondary metabolites involved in defense against herbivores and microorganisms. Fungal cell-wall elicitors and methyljasmonate can induce secondary metabolism in soybean cell cultures by different mechanisms (Enyedi *et al.*, 1992).

Jasmonates have several biological activities, including promotion of the stomata closure (Horton, 1991), acceleration of leaf senescence in oats and barley, pericarp senescence in soybean fruit, induction of tendril coiling in *Bryonia*, and stimulation of stem length and differentiated root system in potato plantlets (Weidhase *et al.*, 1987; Lopez *et al.*, 1987; Falkenstein *et al.*, 1991; Ravnkar *et al.*, 1992; Reinbothe *et al.*, 1992). Some of these effects are apparently mediated by controlling gene expression (Rickauer *et al.*, 1997). Intracellular jasmonates transiently accumulate in cell suspension cultures that have been treated with elicitors implicating a complex physiological role for jasmonates, possibly in the signal transduction system of the defense response (Mueller-Uri *et al.*, 1988; Gundlach *et al.*, 1992).

It has been established that treatment of *in vitro* cultures with exogenous methyljasmonate can elicit the accumulation of several classes of alkaloids (Gundlach *et al.*, 1992; Aerts *et al.*, 1996; Zabetakis *et al.*, 1999; Baldwin, 1999).

Baldwin *et al.* (1996) reported that the inhibition of the octadecanoid pathway (jasmonate biosynthesis pathway) via specific inhibitors leads to inhibition of the endogenous jasmonate level in *Nicotiana sylvestris* and also blocks the nicotine accumulation in response to wounding. Accordingly it was shown that exogenous application of jasmonate to *Nicotiana sylvestris* roots or leaves induces nicotine accumulation (Baldwin *et al.*, 1994, 1996). Since the alkaloid formation is often organ-specific, in species of *Nicotiana* the sites of alkaloid formation are the roots, from where they are exported to other plant organs via the xylem (Wink and Roberts, 1998; Robins, 1998; Suzuki *et al.*, 1999).

As a continuation of our studies on elicitation of the biomass and secondary metabolites *in vitro*, in the present communication we report the establishment of stable hairy root cultures of *Nicotiana* via transformation with *Agrobacterium rhizogenes*. These transformed root cultures are valuable and versatile systems for studying the secondary metabolism. Furthermore, the production of pyridine alkaloids in hairy root cultures of *Nicotiana tabacum* was analyzed by capillary GLC-MS after treatment with potential elicitors, such as methyljasmonate, quercetin and salicylic acid.

## Material and Methods

### *Establishment of transformed root cultures*

Hairy root cultures (HRC) of *Nicotiana tabacum* were initiated by infecting excised root tips with *Agrobacterium rhizogenes* strains 15834, TR 105 or LBA (kindly provided by Prof. Dr. W. Alfermann, Düsseldorf, Germany). Transformed root cultures have been kept in liquid hormone-free MS medium (Murashige and Skoog, 1962) on a gyratory shaker at 110 rpm at 25 °C in an illuminated culture room. Cultures were subcultured into fresh liquid hormone-free MS medium every two weeks for growth and alkaloid production.

### *Analysis of rolC and virC genes by polymerase chain reaction (PCR)*

In order to confirm the transformed nature of the cultures, total DNA was extracted from the hairy root cultures of *Nicotiana tabacum*. PCR (in a DNA thermal cycler; Biometra TGradient) was employed to show the presence or absence of *rolC* and *virC* genes. The PCR conditions were as follows: 96 °C for 2 min, followed by 36 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and finally a 15-min extension at 72 °C. The primers for *rolC* DNA were: 5'-ATGGCTGAA-GACGACCTGTGTT-3' and 5'-TTAGCCGATT-GCAAACCTT G CAC-3' (Oono *et al.*, 1993). The primers for *virC* DNA were: 5'-ATCATTTG-TAGC GACT-3' and 5'-AGCTCAAACCTGCTT C-3' (Sawada *et al.*, 1995).

Agarose gel electrophoresis of the PCR products from transformed and non-transformed root cultures as well as from *A. rhizogenes* was carried out to show the presence or absence of *rolC* and *virC* genes.

### *Elicitation*

HRC of *Nicotiana tabacum* were treated with different inducers, such as methyl-jasmonate (MJ), quercetin (Q) and salicylic acid (SA), to stimulate the accumulation of pyridine alkaloids. Dose-dependent induction experiments were repeated with different concentrations (10, 50, 100, and 200 µM) of MJ in MeOH, Q in DMSO, and SA in H<sub>2</sub>O (Sigma-Aldrich-Chemie, Germany). After 24 h of incubation, HRC were harvested by vacuum filtration, weighed, and kept at -20 °C until extraction and analysis by GLC-MS.

### *Alkaloid extraction*

Plant materials were homogenized in 1 M HCl, filtered, and the acid aqueous solution was basified with concentrated NH<sub>4</sub>OH and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic phase was filtered, dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered again, and concentrated *in vacuo*. The acid-base purification procedure was performed three times. The total alkaloid extract was analyzed by capillary GLC and GLC-MS.

### *Alkaloid analysis by GLC and GLC-MS*

Capillary GLC was performed on a Varian gas chromatograph (3300), equipped with an FID de-

tector and a Spectra Physics Integrator SP4290. Conditions: OV-1 fused silica capillary column (15 m × 0.25 mm); carrier gas, He; detection temperature, 300 °C; injection temperature, 250 °C; split, 1:20; oven temperature program: initial temperature 80 °C, 2 min isothermal, increased at 6 °C min<sup>-1</sup> to 300 °C, then 10 min isothermal.

For GLC-MS, an OV-1 fused silica capillary column (30 m × 0.25 mm) was used coupled to a quadruple Finnigan Mat 4515 mass spectrometer. Conditions: carrier gas, He; injection temperature, 250 °C; split ratio, 1:20; oven temperature program, 80–300 °C at 8 °C min<sup>-1</sup>. EI-MS was conducted at 40 eV and evaluated with the INCOS DATA SYSTEM (Institut für Pharmazie und Molekulare Biotechnologie, Heidelberg, Germany).

## Results

### Confirmation of the transformed nature of the HRC by PCR analysis

The results from agarose gel electrophoresis (Fig. 1) of PCR-amplified products from genomic DNA showed the *rolC* gene in HRC (Tr), but not in non-transformed root cultures (N), and a band corresponding to a fragment of the *virC* gene detected only in the DNA from *A. rhizogenes* (Av).

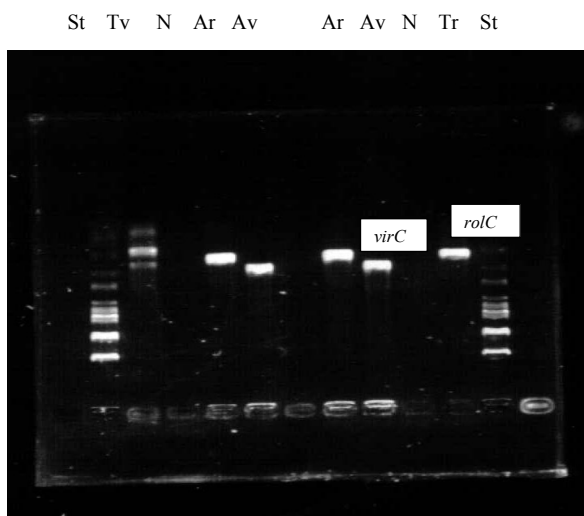


Fig. 1. Agarose gel electrophoresis of PCR products from genomic DNA of a transformed root culture of *Nicotiana tabacum* (T), non-transformed root culture (N), standard DNA (St), and *A. rhizogenes* (A). PCR was performed with *rolC* primers (Tr and Ar) and *virC* primers (Tv and Av).

This finding confirm that the HRC were actually transformed and not contaminated with *A. rhizogenes*.

### Induction of alkaloid accumulation

Accumulation of secondary compounds may in some cases require different manipulations, like a change in cultural conditions or the use of different elicitors to induce the biosynthesis of the secondary products. MJ and SA are involved in signal transduction and induce the transcription of biosynthetic enzymes involved in the formation of defense compounds in plants (Baldwin, 1999). Since MJ can induce the formation of secondary metabolites in other systems, we have tried to stimulate the accumulation of pyridine alkaloids in HRC of *Nicotiana tabacum* by treatment with potential elicitors like MJ and Q.

After 24 h of incubation with MJ, the alkaloids were extracted and determined quantitatively by GLC. The results showed that MJ (50 μM) increased the accumulation of nicotine by approx. 7-fold as compared to the untreated control. The highest stimulation resulted in a nicotine level of 0.58 mg/g fresh weight (FW) after 24 h of incubation (Table I).

The induction can be suppressed by pre-incubation of the cells with SA (Fig. 2). Also SA alone led to a decrease of nicotine contents to less than 1 μg/g FW after incubation for 24 h (Table I). SA has been reported to interfere with the jasmonic acid pathway in other plants (Pena-Cortes *et al.*, 1993; Doares *et al.*, 1995; O'Donnell *et al.*, 1996; Baldwin *et al.*, 1994, 1997).

Also the flavonoid Q, that had been used as an inducer for benzophenanthridine alkaloids in *Sanguinaria canadensis* cultures (Mahady

Table I. Nicotine content in hairy root cultures of *Nicotiana tabacum* after incubation for 24 h with different concentrations of methyljasmonate (MJ), quercetin (Q), and salicylic acid (SA). The values are calculated as the mean ± SD.

Elicitor concentration	Nicotine content [μg/g FW]		
	MJ	Q	SA
Control	79 ± 13	75 ± 11	68 ± 9
10 μM	9.8 ± 1	40.6 ± 11	1.1 ± 0.5
50 μM	580 ± 103	2.3 ± 0.1	1 ± 0.1
100 μM	23 ± 10	2.5 ± 2	0.9 ± 0.2
200 μM	226 ± 79	341 ± 85	0.7 ± 0.1

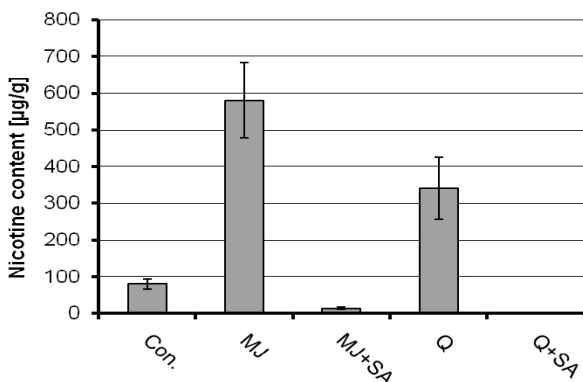


Fig. 2. Effect of salicylic acid (SA) on the induction effect of methyljasmonate (MJ) and quercetin (Q). Con, control. The alkaloid content is represented by nicotine as the major one. The cultures were incubated with the inducers for 24 h. The experiment was performed in triplicate and the bars are the mean  $\pm$  SD.

and Beecher, 1994), tropane alkaloids in *Brugmansia suaveolens* (Zayed and Wink, 2004), and  $\beta$ -carboline alkaloid in *Peganum harmala* (Zayed and Wink, 2005), enhanced the nicotine accumulation approx. 4-fold (Table I). The maximum level was 0.3–0.4 mg/g FW nicotine with 200  $\mu$ M Q. If cultures were pre-treated with SA, this induction was significantly suppressed (Fig. 2). We assume that SA inhibits oxidation steps of the jasmonic acid pathway. This observation indicates that Q probably triggers the pyridine alkaloid formation via the jasmonate induction pathway.

Our experiments with *Nicotiana tabacum* are another example for the power of MJ to induce the formation of alkaloids. Since the production of pyridine alkaloids in *in vitro* cultures is of biotechnological importance, the elicitation via MJ offers a chance to improve this yield.

## Discussion and Conclusion

Many projects were carried out to increase the yields of secondary metabolites in plants and

plant tissue cultures. More recently, it has become apparent that the synthesis of alkaloids, which play a key role in regulating the defense response (Wink and Römer, 1986; Wink, 1988, 1992, 2000, 2007, 2008), can often be elicited by jasmonic acid and its esters. It has also been reported that fungal cell-wall elicitors and MJ can activate the secondary metabolism in soybean cell cultures by different mechanisms (Enyedi *et al.*, 1992). Intracellular jasmonates have been shown to transiently accumulate in cell suspension cultures treated with a yeast elicitor preparation implicating a complex physiological role of jasmonates, possibly in the signal transduction system of the defense response (Mueller-Uri *et al.*, 1988). In cell cultures of *Rauvolfia canescens* and *Eschscholtzia californica*, elicitation of benzophenanthridine alkaloids is associated with the biosynthesis of MJ (Gundlach *et al.*, 1992). It has been established that treatments with exogenous MJ can elicit the accumulation of several classes of alkaloids (Gundlach *et al.*, 1992; Aerts *et al.*, 1996; Zabetakis *et al.*, 1999; Zayed and Wink 2004, 2005). In our current investigation, the analytical results showed that nicotine is the main alkaloid in HRC of *Nicotiana tabacum* accounting for 85–90% of the total alkaloids in MS liquid medium. Induction of alkaloid accumulation in HRC of *Nicotiana tabacum* by different elicitors revealed that the most effective elicitor was MJ with a maximum accumulation of nicotine (0.5 mg/g) followed by Q (0.4 mg/g). This confirmed the reported inductive activity of MJ and Q.

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