Rosmarinic Acid Synthesis in Transformed Callus Culture of *Coleus blumei* Benth.

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Agrobacteria mediated *Coleus blumei* tumour tissues were cultured *in vitro* on MS medium. Sixteen diversified transformed callus cultures were maintained for several years in the absence of plant growth regulators and antibiotics without affecting the growth rate. Rosmarinic acid was detected spectrophotometrically in all tissue lines but in different quantities. The highest rosmarinic acid accumulation detected was 11% of dry tissue mass. The relation between culture growth and rosmarinic acid production was investigated in three callus lines. The lines showed different rosmarinic acid accumulation in relation to their growth rate; it was either parallel or inversely related to the tissue growth. The effects of certain medium constituents on the callus growth and rosmarinic acid accumulation were examined in four tumour cell lines. Addition of 4% or 5% sucrose stimulated rosmarinic acid synthesis and decreased callus growth. Nitrogen reduction to one half or one quarter of initial concentration did not affect rosmarinic acid synthesis and decreased callus growth in three lines, while it increased rosmarinic acid accumulation and callus growth in one line. Addition of 0.1 mg/l Phe stimulated rosmarinic acid production in two lines but had little effect on the rosmarinic acid level in others. Rosmarinic acid production was significantly improved on modified macronutrients, where the Ac2 line produced 16.5 mg of rosmarinic acid per tube (0.2 g of dry wt) after being in culture for 35 days.

Key words: Crown Gall, *Coleus blumei*, Rosmarinic Acid

Introduction

Rosmarinic acid (RA) is a common caffeic acid ester in the plant kingdom, especially among Lamiaceae and Boraginaceae species. It is found in all plant organs and exclusively in vacuoles (Häusler *et al.*, 1993). RA is one of the most prominent secondary compounds in *Coleus blumei* (Lamiaceae). Cell cultures of *C. blumei* accumulate a high amount of RA (Ulbrich *et al.*, 1985; Szabo *et al.*, 1999). RA is biosynthesized via the phenylpropanoid pathway (Petersen *et al.*, 1994). This ester of caffeic acid and 3,4-dihydroxyphenyllactic acid is believed to be a part of the plant defense system against fungal and bacterial infections or predators (Petersen, 1994; Pal Bais *et al.*, 2002). Biological activity of RA is described as antioxidant, antibacterial, antiviral and anti-inflammatory (Cuvelier *et al.*, 1996; Chen and Ho, 1997). Rosmarinic acid is used commercially for food preservation. There are many products on the market containing RA, however, only recently the pure compound has been used as a commercial drug.

Several authors reported that besides intact plants, normal callus cultures and agrobacteria-induced crown galls and hairy roots all synthesize RA (Tada *et al.*, 1996; Murakami *et al.*, 1998; Kochan *et al.*, 1999; Chen *et al.*, 2001; Chen and Chen, 1999, 2000). Nutrient medium composition also affects the RA production. Petersen and Alfermann (1988) proposed CB nutrient medium (modified B5 medium; Gamborg *et al.*, 1968) as the best for RA production in *Coleus blumei* cell culture. Higher sucrose concentrations induce RA synthesis in *Coleus blumei*, *Anchusa officinalis* and *Salvia officinalis* cell cultures (Ulbrich *et al.*, 1985; Su and Humphrey, 1990; Hippolyte *et al.*, 1992; Gartlowski and Petersen, 1993; Martinez and Park, 1993). Addition of phenylalanine and modification of inorganic salts in the nutrient medium for *Lavandula vera* stimulate RA production (Ilieva and Pavlov, 1999; Pavlov and Ilieva, 1999; Pavlov *et al.*, 2000).

In our laboratory 16 transformed *C. blumei* callus lines were established among tumour-induced leaf explants, previously infected with different *Agrobacterium* strains (Bauer *et al.*, 2002).

In this paper the capacity for RA production of transformed callus lines of *C. blumei* is reported. We found a significantly higher level of RA production in transformed callus in comparison to the

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normal callus in our experiments. The patterns of tissue growth and RA accumulation, and the influences of the basal media with different macronutrients (MS and CB), sucrose concentration, phenylalanine, nitrogene, and casein enzymatic hydrolysate on the growth and RA synthesis were analyzed and discussed.

Materials and Methods

Plant material

The initiation and establishment of various transformed lines for RA analysis have been described by Bauer *et al.* (2002). The lines were denoted in dependence on the bacterial genotype used for transformation (*Agrobacterium tumefaciens* B6S3: B; *A. tumefaciens* A281: A; *A. rhizogenes* 8196: 8) and infected plant hybrids (green: z; red: c; variegated: o). These transformed callus lines were subcultured on 20 ml MS medium (Murashige and Skoog, 1962) in tubes (∅ 28 mm). For induction of normal callus on leaf and internodal explants, the MS medium was supplemented with different 6-benzyl-aminopurine and α-naphthaleneacetic acid concentrations (Zagrajski *et al.*, 1997), or with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin. The cultures were incubated at 24 °C under fluorescent light (16 h photoperiod, 80 μE/s^2 intensity) and subcultured every four weeks. Clonal pot plants grew on the window sill in our lab.

Determination of RA concentration

One half of the harvested tissue mass was dried at 105 °C until constant weight was achieved in order to determine the percentage of dry mass. The remainder tissue, which was used for the determination of RA, was extracted with 70% ethanol (1:10) in mortar. Extracts were vortexed, incubated for 10 min at 70 °C, vortexed again, and then incubated for 10 min at 70 °C. Cell residues were settled at 3000 × *g* for 15 min. RA concentration was determined spectrophotometrically at 330 nm (ε = 19,000 l/mol cm).

We detected RA production in leaves of two-year-old pot plants, three-month-old *in vitro* shoots and 33-day-old cultures of transformed and normal callus lines, grown on basal MS medium solidified with 0.8% agar.

Culture parameters and growth kinetics

In order to compare growth and RA production of the lines we followed several phenotypic characteristics of callus lines *e.g.* tissue colour, structure, growth index, dry mass and RA synthesis in tissue.

The specimens of the callus tissue were weighed, and the growth index was determined according to the formula:

$$\text{Growth index at day n} = \frac{\text{Tissue mass at day n} - \text{Tissue mass at start}}{\text{Tissue mass at start}}$$

Dynamics of callus growth and RA production were investigated by inoculating ca. 0.25 g of fresh callus mass onto hormone-free MS medium containing 3% sucrose and 0.8% agar. The investigation was performed throughout the culture cycle until the majority of the tissue died (45 d for Ac1 line, 55 d for 8o4 line and 59 d for Bz1 line). During the whole period, we periodically measured the growth index, percentage of dry wt and RA content in each sample. For each measurement 5 repetitions were performed.

In order to select an optimal nutrient medium for RA production different nutrient medium modifications were tested. We evaluated the effect of CB medium (Petersen and Alfermann, 1988) on RA production, with or without addition of 2 g/l casein hydrolysate. Regarding the MS medium, we evaluated its effect with or without addition of 2 g/l casein hydrolysate and different quantities of Phe, sucrose and nitrogen. Phe was added in concentrations of 0.1, 0.3 or 1.0 g/l and sucrose as 3, 4, 5 or 7% (w/v). The values of nitrogen involved were one-half, one-quarter and one-eighth of the nitrogen concentration normally present in MS medium (1.9 g/l KNO_3 and 1.65 g/l NH_4NO_3). RA content in callus was measured on the 35th day of subculture. All tests were carried out in 6 repetitions. Statistical analysis of data was evaluated by using the DNMR test.

Results and Discussion

Callus growth and RA synthesis

In contrast to the transformed callus, the establishment of the normal callus culture was difficult. After careful selection and cultivation of variegated leaf and internodal explants on MS medium supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin, normal callus lines

were maintained and grew fast as a loose yellow mass. The callus induced on green leaf explants grew slowly in form of a lumpy mass. The same hormonal combination was insufficient for induction and establishment of callus tissue on internodal explants of the green *C. blumei* hybrid and on leaf and internodal explants of the red *C. blumei* hybrid.

The established sixteen transformed callus lines grew on agar-solidified (0.8%) MS medium without plant growth regulators. The tissue colour varied among lines. Six of them were pale green (Bz1, Bz9, Bo9, Ao9, 8z9, 8z12), six were yellowish-brown (Bz4, Bz5, Bc12, Ac2, 8c1, 8c2) and four were ochre (Bo95, Az9, Ac1, 8o4). The lines with the green phenotype grew as a solid compact mass. The ochre and yellowish-brown lines grew as a lumpy (Ac1, 8c1, 8c2, 8o4) or loose cell mass (Bo95, Bz4, Bz5, Bc12, Az9, Ac2). The growth rate and feasibility for RA production of five transformed callus lines and one normal callus line were analyzed on the 33rd day of subculture (Fig. 1). The lumpy and loose cell cultures grew faster than compact lines. RA accumulation in red, green and variegated leaves of *C. blumei* plants grown in pots and in shoots cloned *in vitro* was also analyzed. The red *C. blumei* hybrid accumulated the highest RA amount: leaves from two-year-old pot plants had 6.9% RA in the dry tissue mass and three-month-old *in vitro* shoots had 7.7% RA in the dry tissue mass. Green and variegated *C. blumei* grown in pots accumulated 6.0% and 4.8% RA in the dry tissue mass, respectively, and three-month-old *in vitro* shoots of green and variegated hybrids accumulated 5.5% and 4.2% RA in the dry tissue mass, respectively.

We were not able to establish a relation between either callus structure or colour with the feasibility for RA production, but the appearance of blue pigment on the surface of the callus implied a high percentage of RA in the tissue. Blue pigment is known from cell cultures of *Lavandula* and is identified as a complex of RA and a Fe^{2+} ion (Lopez-Arnaldos *et al.*, 1995). Seven out of sixteen transformed lines accumulated 1.5 to 3% RA of the dry mass, which was the same level of RA in normal callus. The highest amount of RA was 7.5% in the dry mass in line Ac1.

In order to be able to study the relation between culture growth and RA accumulation, both processes were monitored in three transformed lines (Fig. 2). The transformed lines showed a sigmoid growth curve (Fig. 2a) after a surprisingly long lag phase (18 d). Ac1 and 8o4 lines entered the stationary growth phase after 40 and the Bz1 line after 47 d.

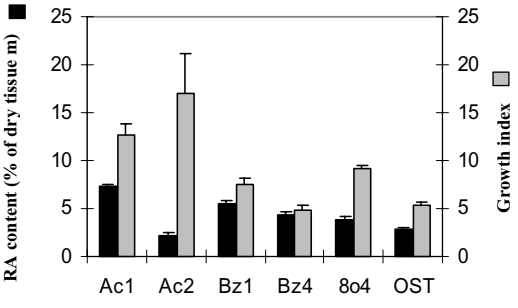

Fig. 1. RA content and growth index of *Coleus blumei* transformed and normal callus lines on the 33rd day of subculture on MS nutrient medium (transformed tissue: Ac1, Ac2, Bz1, Bz4, 8o4) or on MS medium supplemented with 1.0 g/l 2,4-dichlorophenoxyacetic acid and 0.1 g/l kinetin (normal tissue: OST). Standard error bars are shown (n = 5).

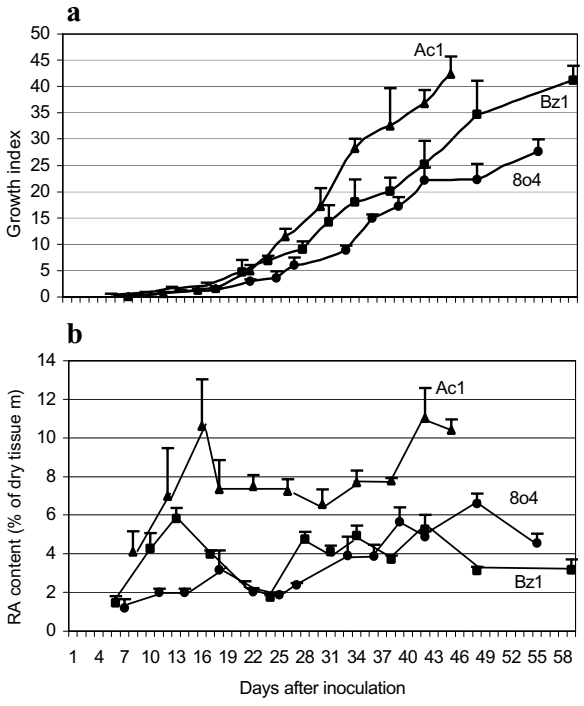

Fig. 2. Growth kinetics (a) and RA content (b) in *Coleus blumei* transformed lines (Ac1, Bz1, 8o4) on MS nutrient medium with 3% sucrose. Standard error bars are shown (n = 5).

The first tissue necroses appeared on the surface of calli in the Ac1 line after 30 d, in the Bz1 line after 34 d and in the 8o4 line after 36 d. After 45 d 90% of Ac1 and 8o4 calli necrotized, however, the part of calli that was in direct contact with nutrient medium still grew.

RA synthesis had 2 peaks in Ac1 and 8o4 lines: the first one at the end of the lag phase of callus growth and the second at the beginning of the stationary phase (Fig. 2b). To be more precise, RA accumulation in the dry mass was 10.6% on the 16th day in the Ac1 line and 11.0% in the stationary phase (42nd day), which represent two highest values. The 8o4 line had a small peak in the lag phase (3.2% RA of the dry mass) and accumulated 6.6% RA of dry mass on the 47th day, which represents the highest value in this line. RA accumulation in Ac1 and 8o4 calli was inversely related to the callus growth, as has been reported for RA accumulation in cell cultures of *C. blumei* (Petersen *et al.*, 1994) and *S. officinalis* (Hippolyte *et al.*, 1992).

RA accumulation in the Bz1 line reached its highest values (5.8% of dry mass) in the lag phase, followed by a decrease in value at the beginning of the exponential phase. However, during the exponential phase RA production was parallel with the callus growth and was around 5% (Fig. 2b). The RA production pattern in the Bz1 line was similar to that described for a *Anchusa officinalis* cell culture (Mizukami and Ellis, 1991) and *Salvia fruticosa* embryogenic tissue (Kintzios *et al.*, 1999).

Induction of RA synthesis

In order to improve the nutrient medium for RA production, the effect of 13 different medium compositions was tested on four transformed lines (Ac1, Ac2, Bz1, 8o4). The amount of RA content in callus was evaluated after 35 d of subculture (Fig. 3). Transformed lines responded differently on different nutrient media. The best basal medium for RA production was CB nutrient medium (Fig. 3a). In comparison with control MS medium, the Ac2 line produced 400% (16.5 mg/tube), the Bz1 line 300% (13.7 mg/tube), the 8o4 line 50% (8.9 mg/tube) and the Ac1 line 30% (9.8 mg/tube) more RA on CB medium. Addition of 2 g/l casein into MS and CB media inhibited the growth of the lumpy 8o4 line and diminished the growth of the Ac1 line and RA synthesis in this line. RA accumulation in the Ac2 line was not significantly changed after addition of 2 g/l casein into MS medium, but was diminished when casein was added into CB medium. Addition of casein into MS nutrient medium stimulated the growth of the compact Bz1 line and RA production in this line, but when added into CB nutrient medium the growth and RA production were diminished in the same line, as in the other lines (Fig. 3a).

A lower concentration of nitrogen in the nutrient medium diminished the growth of the lumpy 8o4, Ac1 and Ac2 lines, but improved the growth of the compact Bz1 line (except when the added concentration of nitrogen was reduced to one eighth of nitrogen concentration otherwise present in the control medium). A nitrogen reduction to one half of the normal value had no significant effect on RA accumulation. The growth of the Ac2 line was considerably suppressed, which resulted in a significantly lower level of RA accumulation on medium in which nitrogen was reduced to one quarter of the normal value. When cultured on media with reduced concentrations of nitrogen, the Ac1 callus was growing somewhat slower, however, RA accumulation was not significantly different in comparison with the control medium. A significantly higher RA accumulation occurred in the Bz1 line on medium with one quarter of the nitrogen concentration. In comparison with the control medium, a significantly lower RA accumulation occurred in all lines (except Ac1 line) when cultured on medium with one eighth of the nitrogen concentration (Fig. 3b).

Increased sucrose concentrations in MS nutrient medium diminished the growth of all lines. Media with 4% and 5% of sucrose stimulated RA accumulation more than MS medium with 3% sucrose (control), except for the Ac2 line. Addition of 7% sucrose in the medium proved to be lethal for the Ac1 and Ac2 lines and suppressed the growth of 8o4 and Bz1 calli considerably, therefore the final effect was a significantly lower RA accumulation (Fig. 3c).

Medium supplemented with 0.1 g/l Phe increased RA level in the Ac2 callus, but had no effect on RA accumulation in 8o4, Bz1 and Ac1 calli. With addition of 0.3 g/l Phe RA accumulation was increased solely in the Bz1 callus. Addition of 1 g/l Phe considerably suppressed the callus growth, therefore RA accumulation decreased in comparison to the control medium (0.0 g/l Phe, Fig. 3d).

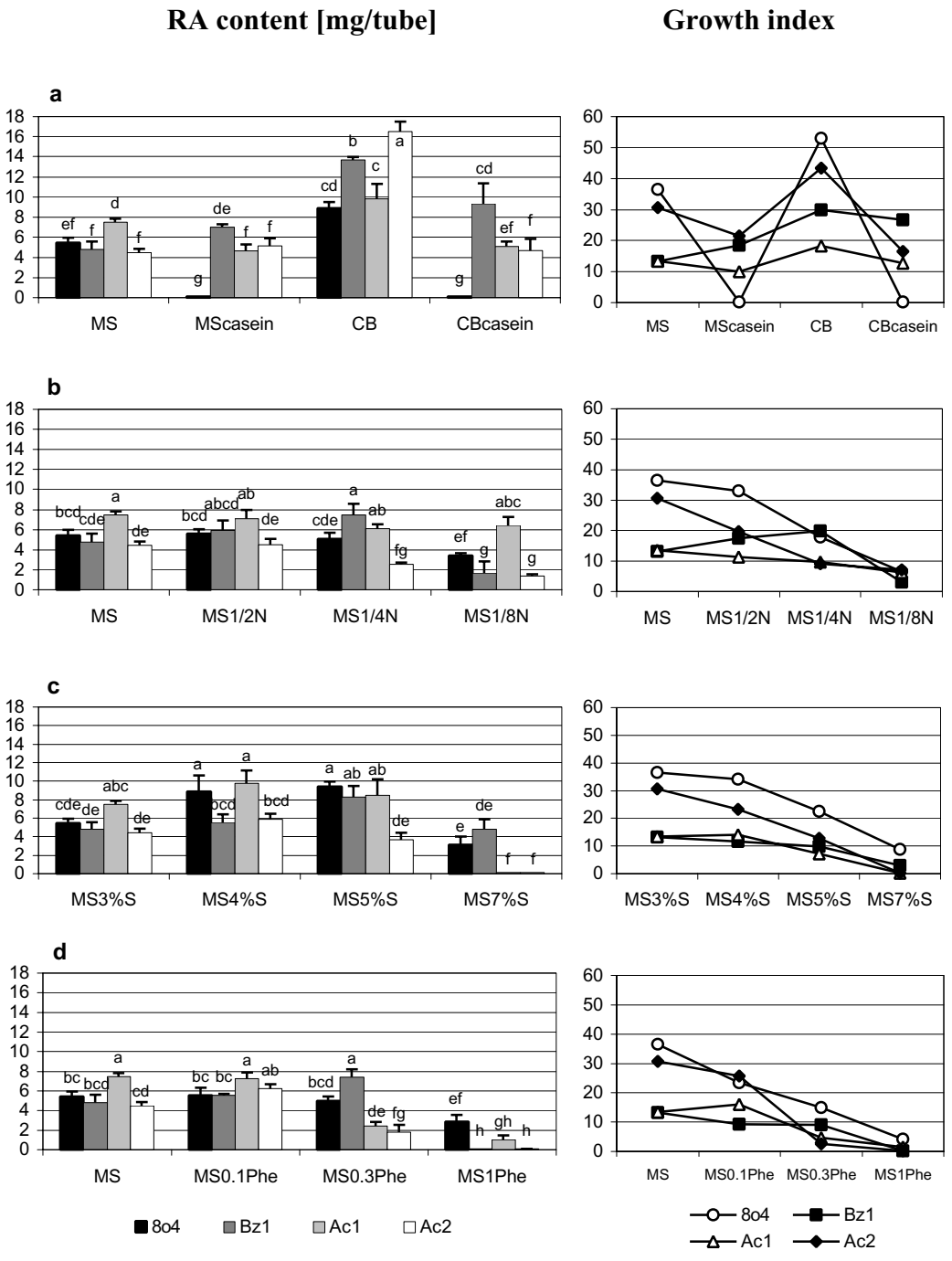

Fig. 3. Effects of media constituents on RA accumulation and growth in 4 transformed *C. blumei* callus lines after 35 d in culture: a) MS and CB nutrient media, MS and CB nutrient media supplemented with 2 g/l caseine hydrolysate; b) MS medium with 1, ½, ¼ and ⅛ × nitrogen concentration; c) MS medium with 3%, 4%, 5% and 7% sucrose; d) MS medium with 0.0, 0.1, 0.3 and 1.0 g/l Phe. Standard error bars are shown (n = 6). Results marked with the same letter are not significantly different.

In conclusion, *C. blumei* transformed callus lines were growing faster and accumulated more RA than normal calli or intact plants. Two patterns of RA accumulation were noticed, either parallel to the growth or inversely related to the growth. Variation of the nutrient medium composition influenced the growth and RA accumulation. The best medium for RA production was CB nutrient medium and the most effective stimulator was sucrose.

It was not difficult to establish transformed *C. blumei* calli, yet isolation of a highly productive tissue line required establishing of a great number of lines and selection of the best one among them.

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