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The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots

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Abstract

The growth, the alkaloid production, as well as the scopolamine/hyoscyamine ratio of two clones of belladonna hairy roots were studied. The effects of nitrate and ammonium concentrations on these cultures were investigated. A rise in ammonium concentration caused the decline of the hairy roots, while nitrate had a marked effect on the alkaloid content. The alkaloid production obtained with 15.8 mM of NO_3^- and 20.5 mM of NH_4^+ was 1.2–1.4 times higher than that obtained when the roots were grown in the standard Murashige and Skoog medium (MS medium, 39.5 mM of NO_3^- and 20.5 mM of NH_4^+). The nitrate and ammonium concentrations in the culture medium also had a strong influence on the scopolamine/hyoscyamine ratio. When nitrate or ammonium concentrations were raised, that ratio also was increased 2–3-fold. The hairy root clones originating from transformations with two distinct strains of *Agrobacterium* had similar responses.

Keywords: *Atropa belladonna*; Ammonium; Hairy roots; Hyoscyamine; Nitrate; Scopolamine

1. Introduction

Hairy roots of *Atropa belladonna* (Solanaceae) are a fast-growing and reliable tropane alkaloid-producing material. They are known for their high secondary product content (Jung and Tepfer, 1987; Parr et al., 1990). Hyoscyamine is the major alkaloid and scopolamine is usually in smaller supply, which makes it the most valuable and the

Abbreviations: C8, *Atropa belladonna* hairy root clone transformed with the genes *rolA*, *rolB* and, *rolC*; C4, *Atropa belladonna* hairy root clone transformed with *Agrobacterium rhizogenes* 15834; d.w., dry weight; MES, 2-[*N*-morpholino] ethanesulfonic acid; Rt, retention time.

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most demanded of the tropane alkaloids (Hashimoto et al., 1993). As hairy root cultures are able to keep a stable production of alkaloids over long periods of subculturing (Maldonado-Mendoza et al., 1993), they are considered as an interesting option for the study of alkaloid biosynthesis (Parr, 1989).

Nutritive factors like the nitrogen supply are important parameters influencing alkaloid production (Berlin et al., 1985). Growth and tropane alkaloid production of *Atropa belladonna* hairy roots are influenced by the composition of the culture medium (Aoki et al., 1997). In *Datura quercifolia* hairy roots, the growth and the tropane alkaloid content are depressed by highly concentrated media (Dupraz et al., 1993). The dilution of the culture media and the reduction of the nitrogen concentrations enhance growth and alkaloid content in hairy root cultures of Solanaceae (Payne et al., 1987; Parr et al., 1990). The nitrogen concentration and the carbon/nitrogen ratio of the culture medium often influence the synthesis of alkaloids (Payne et al., 1987; Sugimoto et al., 1988). In vitro Solanaceae cultures are usually grown on media containing both nitrate and ammonium as nitrogen sources. The NH_4^+/NO_3^- ratio strongly influences the growth of plant cell cultures (Veliky and Rose, 1973). However all these effects of the culture medium are different from one clone to another, this makes the optimization of culture conditions very complicated (Aoki et al., 1997).

In our paper, the nitrate and ammonium concentrations of MS medium were modified. Their effects on the growth, the alkaloid (hyoscyamine and scopolamine) content and production as well as the scopolamine/hyoscyamine ratio of belladonna hairy roots were investigated. Two root clones originating from transformations with two distinct types of *Agrobacterium* were compared in order to bring out general tendencies.

2. Materials and methods

2.1. Plant material

Two root clones initiated from *Agrobacterium*-mediated leaf-disk infections were used (Bonhomme, 1997). The C8 clone was transformed with a recombinant *Agrobacterium tumefaciens* strain holding three genes of the *A. rhizogenes* Ri T-DNA [*rolA*, *rolB* and *rolC*; (Spena et al., 1987)]. Gene insertion was checked by PCR and Southern-blotting of the transgenes. The C4 clone was obtained after infection with a wild strain of *A. rhizogenes* 15834.

2.2. Culture conditions

The hairy roots were maintained in 50 ml liquid modified MS medium (3% sucrose and 0.5 gl^{-1} filter sterilized cefotaxime) in 100 ml Erlenmeyer flasks. pH was adjusted to 5.8 and supplemented with MES (0.5 gl^{-1}). The cultures were incubated in the dark at 22°C on a rotatory shaker at 110 rpm. One gram of root tips (1 g fresh weight $\approx$ 80 mg d.w.) were inoculated. The roots were harvested after a period of 4 weeks, and then blotted to dry on filter paper before they were weighed.

2.3. Nitrate and ammonium concentrations

Three levels of nitrate (15.8, 39.5, 98.75 mM) and three levels of ammonium (8.2, 20.5 and 51.25 mM) concentrations were tested (ammonium sulfate and potassium nitrate) (Veliky and Rose, 1973). MS liquid medium (39.5 mM nitrate and 20.5 mM ammonium) was taken for reference (Murashige and Skoog, 1962), the other media were modified MS media. Every experiment was repeated twice.

2.4. Tropane alkaloid extraction

Freeze-dried tissues were ground and tropane alkaloids were extracted twice from samples weighing 100 mg, with 50 ml of $CHCl_3$ – 30% NH_4OH (49:1) under reflux. Combined extracts were dried over Na_2SO_4, filtered and evaporated under reduced pressure in a thermostatic bath (45°C). The residue was redissolved in the HPLC solvent and analysed into replicate sets. Only

traces of alkaloids were detected in the culture medium of hairy roots and they were not taken into account for the study.

2.5. Alkaloid analyses (hyoscyamine and scopolamine)

Alkaloids were assayed by quantitative HPLC (Fliniaux et al., 1993). The stationary phase was a C18 Nucleosil (250×4.6 mm) column. The elution solvent was composed of water–acetonitrile–phosphoric acid–triethylamine (83.4:16.5:0.1:0.02, v/v). A spectrophotometric UV detection was used ($\lambda = 204$ nm). The calibration was made with standard scopolamine hydrochloride ($Rt = 6.2 \pm 0.5$ min) and L-hyoscyamine ($Rt = 8.3 \pm 0.5$ min). Every sample was assayed in duplicate. The alkaloid production was determined by both biomass production and alkaloid content:

$$\underset{\text{mg flask}^{-1}}{\text{Alkaloid Production}} = \underset{\%\ \text{d.w.}}{(\text{Alkaloid Content})} \times \underset{\text{mg flask}^{-1}}{(\text{Dry Weight})} \quad (1)$$

3. Results

3.1. Effects of the nitrate and ammonium concentrations on growth

When the hairy roots were grown in the MS medium (20.5 mM of NH_4^+ and 39.5 mM of NO_3^-), clone C8 yielded more d.w. than C4 (423 and 364 mg flask^{-1}, respectively) (Table 1). The effects of nitrate and ammonium on the production of biomass was clearly visible even before weighing the roots. A decline of d.w. to less than 204 mg flask^{-1} was observed for both clones when nitrate concentration was raised in the medium (98.75 mM of NO_3^-). The same observation was made when the ammonium concentration was raised (51.25 mM of NH_4^+). The d.w. was also lower than in the MS medium when both nitrate and ammonium concentrations were reduced in the culture medium (8.2 mM of NH_4^+ and 15.8 mM of NO_3^-). The highest yield of d.w. was obtained with the same nitrate concentration as in the MS medium (39.5 mM of NO_3^-) but with a reduced ammonium concentration (8.2 mM). In these conditions, the d.w. of C8 was 429 mg, for C4 it was 562 mg. That increase was more significant for clone C4 than it was for C8. For C4, the yield of d.w. obtained in these conditions was 1.5 times higher as compared to that obtained with the MS medium. Lower concentrations (below 15.8 mM for nitrate and below 8.2 for ammonium) showed the limits of this tendency.

3.2. Effects of the nitrate and ammonium concentrations on alkaloid contents and production

When the roots were grown in the MS medium, the alkaloid content of clone C4 was higher than that of C8 (0.28 and 0.23% of the d.w., respectively) (Table 2). The alkaloid content decreased in the roots of both clones when the ammonium concentration was reduced to 8.2 mM, except in the case where the nitrate concentration was also reduced in the medium. In that case the alkaloid content remained unchanged (0.24% d.w. for clone C8) or even became higher (0.33% d.w. for C4) than in MS conditions. An overall increase of the alkaloid content of the roots was also observed when the nitrate concentration was re-

Table 1
Influence of the initial nitrate and ammonium concentrations in the culture medium on the yield of dry weight (mg flask^{-1})[a]

Hairy root line	$[NH_4^+]$ (mM)	$[NO_3^-]$ (mM)		
		15.80	39.50	98.75
Line C8	8.20	381	429	418
	20.50	413	423	161
	51.25	143	144	81
Line C4	8.20	339	562	557
	20.50	492	364	204
	51.25	223	160	93

[a] The data are means of two values.

Table 2
Influence of the initial nitrate and ammonium concentrations in the culture medium on the alkaloid content (% of the d.w.) and production (mg flask^{-1})[a]

Hairy root line	$[NH_4^+]$ (mM)	$[NO_3^-]$ (mM)					
		15.80		39.50		98.75	
		%	mg flask^{-1}	%	mg flask^{-1}	%	mg flask^{-1}
Line C8	8.20	0.24	0.92	0.18	0.79	0.13	0.56
	20.50	0.27	1.11	0.23	0.77	0.20	0.32
	51.25	0.33	0.47	0.18	0.26	0.21	0.21
Line C4	8.20	0.33	1.13	0.18	1.03	0.23	1.27
	20.50	0.27	1.32	0.28	1.07	0.42	0.86
	51.25	0.33	0.73	0.31	0.49	0.20	0.19

[a] The data are means of four values.

duced to 15.8 mM in the medium (Table 2). This tendency was confirmed with a lower nitrate concentration (6.4 mM). In this case, the alkaloid content of clone C4 increased to 0.4% d.w. (data not shown).

The culture conditions providing a maximal alkaloid production were the intersection of conditions (nitrate and ammonium concentrations) which enabled a good growth and high alkaloid accumulation. In the reference conditions (MS medium), clone C4 had a total alkaloid production higher than that of C8 (1.07 and 0.77 mg flask^{-1}, respectively) (Table 2). For both clones C8 and C4, the maximal alkaloid amounts were obtained when the level of nitrate was reduced to 15.8 mM of NO_3^- (Table 2). These amounts were 1.11 and 1.32 mg flask^{-1}, respectively, and they were 1.4 and 1.2 times higher than when the roots were grown in the MS medium. This way, a reduction of the nitrogen level improved the global alkaloid production. With lower concentrations (6.4 mM of NO_3^- and 3.3 mM of NH_4^+) the alkaloid production was slightly declining (around 1.2 mg flask^{-1}, data not shown) mainly due to the poor biomass.

3.3. Effects of the nitrate and ammonium on the scopolamine/hyoscyamine ratio

The scopolamine/hyoscyamine ratio of clone C4 was higher than that of C8 in the MS medium (0.29 and 0.25, respectively) (Table 3). When the nitrate concentration was reduced to 15.8 mM, this ratio was the double of that obtained in MS medium. When the ammonium concentration was raised from 20.5 to 51.3 mM, the scopolamine/hyoscyamine ratio also increased to reach its highest values wich were 1.39 and 1.25 for C4 and C8, respectively.

4. Discussion

Clones C4 and C8 are totally independant because they originate from different plant materials, different bacterial strains and different transformation events. C8 was transformed with a recombinant bacterial strain holding *rolA*, *rolB*, and *rolC*

Table 3
Influence of the initial nitrate and ammonium concentrations in the culture medium on the scopolamine/hyoscyamine ratio[a]

Hairy root line	$[NH_4^+]$ (mM)	$[NO_3^-]$ (mM)		
		15.80	39.50	98.75
Line C8	8.20	0.14	0.29	0.17
	20.50	0.50	0.25	0.43
	51.25	0.60	1.25	1.10
Line C4	8.20	0.18	0.29	0.03
	20.50	0.50	0.29	0.20
	51.25	0.60	1.39	1.10

[a] The data are means of four values.

genes. The combination of these three genes is enough to obtain the morphological characteristics of the hairy roots (Spena et al., 1987). C4 and C8 showed different growth and alkaloid contents. Despite these quantitative differences, the influence of nitrate and ammonium did not change from one clone to the other. The physiological response of C8, transformed with *rolA*, *rolB*, and *rolC* genes, to nitrate and ammonium concentrations was the same to that of C4, wich was transformed with the wild *A. rhizogenes* strain. This result implies that the combination of *rol A*, *rolB*, and *rol C* genes is sufficient to obtain hairy roots with, not only the same morphology, but also the same physiological response to nitrate and ammonium as the hairy roots obtained with the wild *A. rhizogenes* strain.

Growth and alkaloid accumulation were influenced by both nitrate and ammonium concentrations. The same effects were observed on both clones and general tendencies were uncovered.

We have shown that the highest yields of d.w. were obtained with reduced levels of ammonium. Our results are consistent with that of Yamamoto and Kamura who reported that ammonia salts strongly inhibited the growth of *Bupleurum falcatum* L. roots (Yamamoto and Kamura, 1997). Ammonium is very diffusive and it easily accumulates into the tissues, becoming very toxic if not immediately metabolized (Richter, 1993). When the ammonium concentration in the medium is low, most of the accumulated ammonium is metabolized by the cells, while in the case where the ammonium concentration is too high, only a small part can be metabolized and the excess has inhibitory effects on the cell metabolism. Another consequence of the accumulation of ammonium could be a direct or indirect repressive effect on the nitrate assimilation (Crawford, 1995).

The effects of nitrate concentration on the hairy roots was more evident at the level of the alkaloid contents. A reduction of the nitrate concentration in the culture medium lead to increased alkaloid contents in the hairy roots of belladonna. The concentrations providing a maximal alkaloid production were the intersection of conditions (nitrate and ammonium concentrations) which enabled a good growth and a high alkaloid accumulation.

The scopolamine/hyoscyamine ratio was also modulated by the composition of the culture medium. It was strongly enhanced by a raise of the concentrations of nitrate and ammonium. It was increased (2–3-fold) when the concentrations of nitrate and ammonium were raised. This result implies that the concentrations of these two nutrients can play an important role in the regulation of scopolamine biosynthesis.

Our results suggest that ammonium plays an important role during the first days of culture, i.e. just before or during the rapid growth period of the hairy roots, while nitrate influences later events in the life of the cultured tissues, like the biosynthesis of the tropane alkaloids. This can be related to some observations of Hilton and Wilson who showed that ammonium was totally and rapidly removed from the medium in the first days of culture preceding the exponential growth, while only a part of nitrate was uptaken, later and gradually (Hilton and Wilson, 1995).

5. Conclusion

In conclusion, quantitative and qualitative changes in d.w. and tropane alkaloid yields of belladonna hairy roots were achieved by a modification of the nitrate and ammonium concentrations in the medium. Nitrate and ammonium had distinct effects on growth or alkaloid accumulations. Ammonium had a strong influence on growth, while nitrate had a clear influence on the alkaloid content and on the scopolamine/hyoscyamine ratio. All these effects were the same for clones C8 and C4 which originated from transformation with two distinct types of *Agrobacterium*.

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