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Modification of the embryogenic response of *Coffea arabica* by the nitrogen source

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Abstract

Somatic embryogenesis was induced in coffee from *in vitro* cultured plants as starting material and the faster response obtained allowed lines from selected plants to be generated more quickly. In contrast to other systems, where embryos take 2 or 3 months to develop, globular embryos were obtained after 3 weeks. The optimum nitrogen concentrations for embryogenesis were between 3.75 and 15 mM nitrogen with a nitrate/ammonium molar ratio of 2:1 or 1:2.

Introduction

Nitrate and NH_4^+ are important for plant differentiation (Halperin & Wetherell 1965, Reinert *et al.* 1967) and for promoting embryo development (Joy *et al.* 1996). The total nitrogen content, the nitrate/ammonium ratio and the inorganic/organic ratio, as well as the nature of the nitrogen source, have a considerable effect on the response of explants to somatic embryogenesis induction (Wetherell & Dougall 1976, Mordhorst & Lörz 1993, Grimes & Hodges 1990).

Cultures maintained on NO_3^- as the sole nitrogen source do not form embryos when placed under inductive conditions (Joy *et al.* 1996). A reduced nitrogen source is always required, at least as a supplement of nitrate, for rapid growth and *in vitro* embryogenesis of cultured wild carrot tissue (Wetherell & Dougall 1976). There is an absolute requirement in alfalfa for the ammonium ion during embryo induction and differentiation (Meijer & Brown 1987).

Growth, embryogenesis, the morphology of the regenerated plants and plant regeneration from *in vitro* cultured tissues can be manipulated independently through the nitrogen supply (Mordhorst & Lörz 1993, Grimes & Hodges 1990). To increase the yield of these processes, manipulation of the nitrogen source, or the addition of conditioned medium into the culture medium can be used as a biotechnological tool (Chung *et al.* 1992).

However, the exact role and the mechanism by which the nitrogen source influences the differentiation process is still unknown. For instance, nothing is known of this process in tropical plants, such as coffee, which has its own characteristic somatic embryogenesis process. The aim of this research was to study the effect of the nitrogen source on coffee somatic embryogenesis.

Materials and methods

Plant material

Seeds of *Coffea arabica* cultivar Caturra Rojo were collected in Chiapas, Mexico. Seeds were washed and soaked for 24–48 h in sterile distilled water, disinfected with 6% $NaClO_3$ for 20 min and finally rinsed with sterile water. The MS (Murashige & Skoog 1962)

medium was used for zygotic embryo germination, and was augmented with thiamine (29.6 μM), myo-inositol (0.555 μM), biotin (0.41 μM), L-cysteine (0.15 μM), glucose (166.48 mM), naphthalene acetic acid (NAA, 0.53 μM), kinetin (Kin, 2.32 μM) and Gelrite (0.25%) and the pH was adjusted to 5.7 before autoclaving (20 min, 110 °C). Ten embryos were placed in each Magenta plastic box and cultured at 25 °C with a photoperiod of 16 h/8 h.

Plantlet culture

Plantlets were grown in Magenta boxes, containing 40 ml supplemented MS medium at 25 °C with a photoperiod of 16 h/8 h and were transferred to fresh medium every 90 days.

Somatic embryogenesis induction

Leaf fragments (0.25 cm^2) were cultured in the medium previously described by Yasuda *et al.* (1985) with Gelrite (0.25%). Culture conditions were a photoperiod of 16 h/8 h and at 25 °C.

Results and discussion

Within the first two weeks after leaf explants were put in the Yasuda culture medium scarring was observed at the wounded edges. Between the third and fourth week, globular somatic embryos were observed on the edges of explants.

Nitrogen concentration played a crucial role in embryo induction as elevated levels of nitrate and ammonium decreased culture embryogenecity. As the total nitrogen in the medium increased, the response to somatic embryogenesis decreased (Figure 1). Nitrogen at 60 and 30 mM had a strong inhibitory effect while between 4 and 15 mM gave a response higher than 70% and reached values near to 90%.

When counted 120 days after the start of the experiment, the number of embryos per explant and the total embryos per experiment varied as a function of total nitrogen content. The presence of nitrogen was indispensable for an embryogenic response. When the total nitrogen source increased, the number of embryos per explant and the total embryos per experiment decreased. The response was almost zero when 60 mM of nitrogen was used (Table 1).

Since the molar ratio of oxidized nitrogen to reduced nitrogen can modify the differentiation status of the tissues, several nitrate/ammonium molar ratios were tested at 15 mM of total nitrogen. The use of either nitrate or ammonium as nitrogen sources, at a nitrate/ammonium molar ratio of 1:0, produced a response around 50% (Figure 2). However, the response was at a maximum when the nitrate/ammonium molar ratio was 2:1 or 1:2. The maximum response with the 2:1 molar ratio was reached 60 days from the start of the experiment, whereas for the 1:2 molar ratio the same maximum values were reached 60 days later.

The number of embryos per explant and total embryos per experiment also changed as a function of the nitrate/ammonium molar ratio when counted 120 days

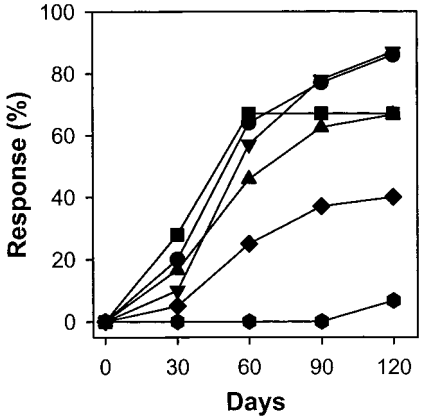

Fig. 1. Effect of the total nitrogen source on direct somatic embryogenesis induction in *C. arabica*, 120 days after induction. (●) 3.75 mM, (■) 7.5 mM, (▲) 9 mM, (▼) 15 mM, (♦) 30 mM and (●) 60 mM. The nitrate/ammonium molar ratio was kept constant at 2:1. In all experiments, 20 explants per treatment were used and each experiment was repeated three times.

Table 1 Effect of nitrogen source on direct somatic embryogenesis induction, total embryos per explant and total embryos per experiment in *C. arabica*, 120 days after induction. In all experiments, 20 explants per treatment were used and each experiment was repeated three times. An ANOVA was performed and the media is shown. The standard deviation is show in parenthesis. Similar superscript letters correspond to media with no statistical significance.

Nitrogen concentration (mM)	Response (%)		Total embryos		Embryos/explant	
0	1.67	$(2.89)^d$	2	$(2)^c$	0.18	$(0.08)^c$
3.75	86	$(7.37)^a$	129	$(20.07)^a$	9.20	$(4.70)^a$
7.5	65	$(2.64)^b$	54	$(4.58)^b$	3.60	$(1.65)^b$
9	66	$(4.16)^b$	85	$(5)^b$	3.54	$(0.84)^b$
15	61.5	$(2)^a$	88	$(15.1)^b$	5.27	$(0.66)^b$
30	40	$(4)^c$	69	$(16.52)^b$	4.60	$(1.25)^b$
60	6.7	$(1.53)^d$	1	$(1)^c$	0.06	$(0.04)^c$

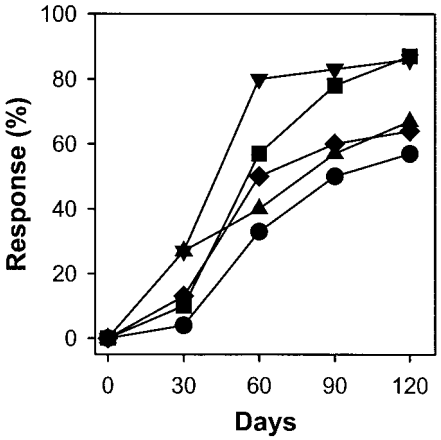

Fig. 2. Effect of different nitrate/ammonium molar ratios on direct somatic embryogenesis induction in *C. arabica*, 120 days after induction. (■) 10:5, (▼) 5:10, (▲) 7.5:7.5, (♦) 0:15, and (●) 15:0. The total nitrogen source was constant at 15 mM. In all experiments, 20 explants per treatment were used and each experiment was repeated three times.

Table 2. Effect of different nitrate/ammonium molar ratio on direct somatic embryogenesis induction, total embryos per explant and total embryos per experiment in *C. arabica*, 120 days after induction. In all experiments, 20 explants per treatment were used and each experiment was repeated three times. An ANOVA was performed and the media is shown. The standard deviation is shown in parenthesis. Similar superscript letters correspond to media with no statistical significance.

NO_3:NH_4 (mM:mM)	Response (%)		Total embryos		Embryos/explant	
15:0	57	$(4.58)^b$	67	$(6.08)^c$	4.47	$(0.55)^b$
10:5	87	$(3)^a$	118	$(7.21)^a$	7.90	$(2.27)^a$
7.5:7.5	67	$(2.64)^b$	72	$(5.29)^c$	5.14	$(1)^b$
5:10	86	$(7.54)^a$	100	$(2)^b$	6.67	$(0.58)^a$
00:15	64	$(5)^b$	119	$(10.54)^a$	8.5	$(0.50)^a$

after the beginning of the experiment. When the ammonium was absent from the medium, only 4 embryos per explant and a maximum of 66 embryos per experiment were obtained. With only ammonium as the nitrogen source, 8 embryos per explant and around 120 total embryos per experiment were obtained, whereas only 65% of the explant formed embryos (Table 2). In coffee, as in carrot (Wetherell & Dougall 1976) and alfalfa (Meijer & Brown 1987) there was an absolute requirement for ammonium in the culture medium for an optimal response to somatic embryogenesis.

Until now there has been no difference in the development of plants coming from somatic embryos produced with different amounts of nitrogen and different nitrate/ammonium ratios (data not shown). This contrasts with rice, in which the morphology of the regenerated plants was strongly influenced by the nitrogen ratio (Grimes & Hodges 1990).

To summarize, we modified the protocol described by Yasuda *et al.* (1985) to create a model that allowed us the use of *in vitro* cultured plants as starting material and the faster response obtained allowed the generation of lines from selected plants more quickly. This model will also allow experiments in coffee that previously were difficult to carry out because of the long somatic embryogenesis protocols.

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