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Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by $AgNO_3$

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Abstract. In order to evaluate the impact of ethylene in maize tissue culture, silver nitrate has been used as an inhibitor of ethylene action. Type II callus initiation rate was improved when immature embryos were cultured on a modified Murashige & Skoog medium containing various concentrations of silver nitrate (5, 10, 20 $mg\,l^{-1}$). Regeneration ability of calli initiated and maintained in presence of silver nitrate was enhanced. No modification of callus growth rate neither of ethylene production has been detected.

Introduction

Since the first experiments of Green & Phillips [6], numerous authors have reported plant regeneration from tissue cultures of maize lines via somatic embryogenesis.

Tissue culture of maize is capable to produce two different types of embryogenic calli [7, 16]: a compact organized and slow-growing callus (type I) and a soft, friable and fast-growing one (type II). Type II callus allows the establishment of finely dispersed embryogenic suspension cultures [8] from which the isolation of totipotent protoplasts of maize have been performed [11, 19, 22]. Unfortunately the production of type II callus arises at low frequency and only for specific genotypes (A188, Dekalb XL 82, B73).

Successful application of biotechnology in maize improvement will largely depend on our ability to induce easily type II callus. In the past, several pathways have been explored in order to increase the production of type II callus: selection of genotypes, use of particular explants such as immature embryos [6] and modification of the culture medium with complex organic additives [1, 8]. No study has emphasized gaseous environment effects for the obtention of type II callus. Among the most important gases, ethylene

(C_2H_4) has a predominant role on growth and development of plants [15, 23]. In order to evaluate the impact of C_2H_4 in our tissue culture, we have used silver nitrate ($AgNO_3$) as an inhibitor of ethylene action [3]. Silver nitrate has been shown to promote regeneration and somatic embryogenesis in tobacco [18], hevea [2] and several monocotyledoneous plants such as pearl millet, wheat [18] and maize [20].

Our experiments using immature corn embryos describe the effect of $AgNO_3$ on initiation, growth and regeneration of type II callus. They try to connect those effects with the presence of ethylene in the tissue cultures.

Materials and methods

Seeds of *Zea mays* L. line A188 were obtained from Limagrain company. Plants were grown in greenhouse in summer 1987. Two to three weeks after controlled self-pollination, ears were harvested and surface-sterilized for 15 min in a 8% hypochlorite solution and then rinsed in three changes of sterile water. Young embyros (1–2 mm length) were aseptically removed from the immature kernels and placed embryo axis in contact with the medium, on the basis of 16 embryos per Petri dish (9 cm diameter, 25 ml medium). For all experiments, the immature embryos from each ear were randomly distributed between the media used.

Embryos were cultured on modified Kamo's medium [12]: Murashige & Skoog (MS) basal salts [17] with added (in $mg\,l^{-1}$) glycine 2.0, thiamine-HCl 0.5, pyridoxine-HCl 0.5, nicotinic acid 0.5, myo-inositol 100, L-asparagine 150, 2,4-dichlorophenoxyacetic acid (2,4-D) 1.0, and (in $g\,l^{-1}$) sucrose 20.0, agarose 6.0. $AgNO_3$ was added at various concentrations: 0 (MS_0), 5.0 (MS_5), 10.0 (MS_{10}), or 20.0 (MS_{20}) $mg\,l^{-1}$. The pH was adjusted to 5.8 with KOH before autoclaving (118 °C; 0.8 bar; 20 min). All cultures were incubated at 24–25 °C, with a photoperiod of 16 h light (1.4 $\mu E\,m^{-2}s^{-1}$), 8 h dark.

Two to three weeks after plating, type I calli were scored, soft friable embryogenic calli were excised and subcultured every 15 days on the same medium. The stabilized type II calli were maintained on the basis of one callus line (i.e. clone) per immature embryo of A188.

Plants were regenerated following the procedure of Green [8]. Sections of type II calli were subcultured on a maturation medium: MS_0 containing 6% (w/v) sucrose and devoid of 2,4-D. Two to three weeks later, embryoids which appeared to be mature were aseptically removed from the callus and transferred to regeneration medium, MS_0 containing 2% sucrose and no 2,4-D, and cultured under light. Plantlets 2–5 cm long were transferred to

culture tubes (2.5 × 15 cm) containing fresh regeneration medium in order to allow their development. When plantlets reached a height of 7–10 cm, with a well-developed rooting system (Fig. 3), they were scored as regenerated plants and transferred to jiffy pots in the greenhouse.

The measurements of callus growth and ethylene concentration were performed with the same clone. This clone has been randomly retained among the stabilized callus lines initiated on MS_0 medium. Growth measurements were done on calli subcultured in Petri dishes (10 calli, 25 mg FW each, per dish). Ethylene measurements were performed with calli (4 calli, 25 mg FW each) cultured in hermetic serum bottles (vol. 250 ml, 30 ml medium) from which 1 ml gas was taken for chromatography (Delsi Instrument, Serie 330 with a porapak column and flame ionization detector).

Results

Callus initiation

Silver nitrate did not modify the frequency of embryos producing type I callus (Table 1). Routinely 5–10% of the immature embryos cultivated on our MS_0 medium and under our culture conditions, produced type II callus. All the concentrations of $AgNO_3$ increased the production of type II callus 4 to 6-fold (Table 1). For all the experiments 5–10 mg l^{-1} appeared to be the optimal level of silver nitrate concentration for the initiation of friable embryogenic callus.

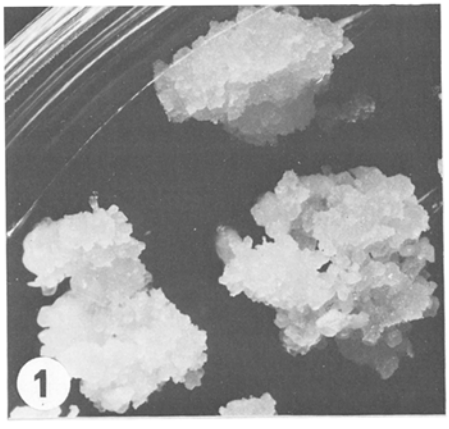
Whatever the silver nitrate concentration used, no modification of the morphological aspect of type II calli has been observed (Figs. 1 and 2).

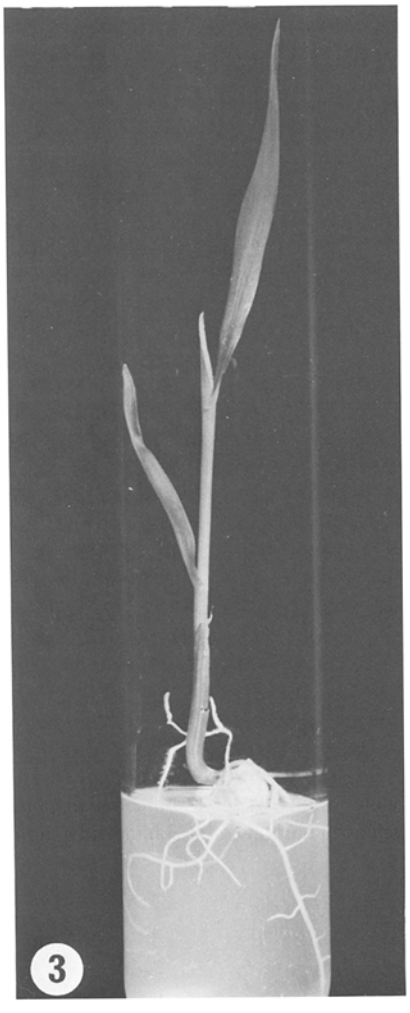
Table 1. Effect of $AgNO_3$ on frequencies of type I and type II calli initiation.

$AgNO_3$ concentration in initiation medium (mg l^{-1})	Number of embryos cultured[1]	Type I callus (%)	Type II callus (%)
0	141	63.8 a[2]	4.9 a
5	130	67.7 a	26.9 b
10	151	66.9 a	34.0 b
20	147	68.7 a	23.8 b

[1]The embryos were cultured from 8 self-pollinated ears of A188 line. Each value is the total of 3 independent experiments.

[2]Entries within column followed by the same letter are not significantly different at $P = 0.05$ by two-way analysis of variance and multiple range test.





Figs. 1–3. (*1, 2*) Morphology and histology of soft friable embryogenic callus (type II) with somatic proembryos onto callus surface (MS_5 medium). (*3*) Plantlet obtained from embryo after maturation stage on MS_0 medium devoid of 2,4-D.

Callus growth and ethylene measurements

Silver nitrate did not modify the growth rate of the selected callus line cultivated in Petri dishes (Fig. 4). There was a general increase of heterogeneity between the calli after 18 days of culture due to the necrosis of several calli. This did not permit any difference in callus growth between the various concentrations of $AgNO_3$ to be observed.

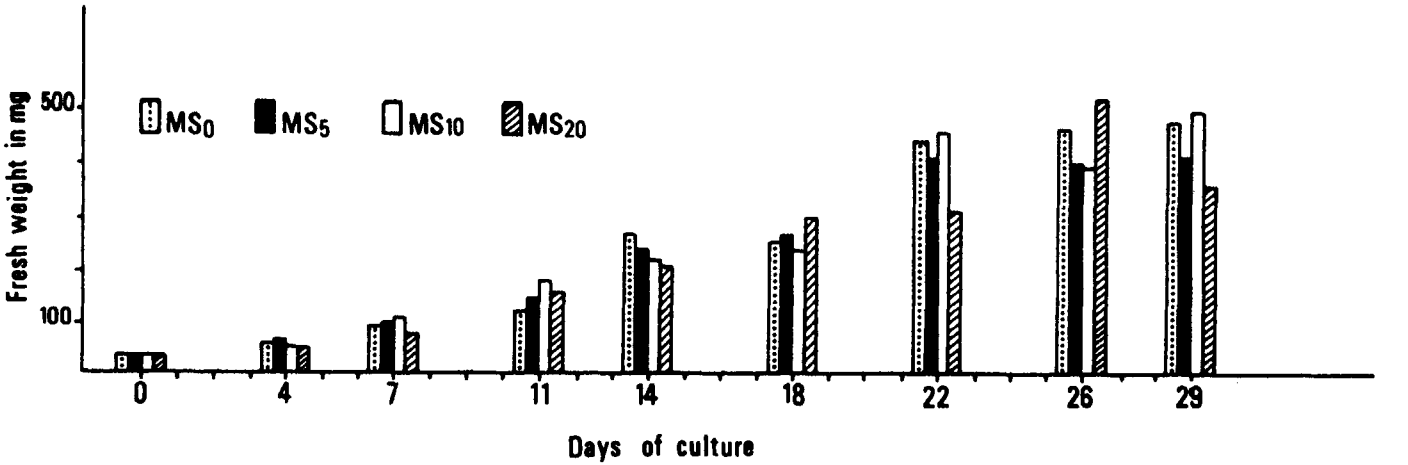


Fig. 4. Effect of $AgNO_3$ on the growth of one type II callus line. Each column is the mean weight of 20 to 80 calli from two independent experiments.

Table 2. Effect of $AgNO_3$ on the concentration of C_2H_4 present in the culture environment of type II calli subcultured since 15 days in hermetic serum bottles.

$AgNO_3$ concentration in culture medium ($mg\,l^{-1}$)	C_2H_4 concentration[1] ($\mu l\,l^{-1}$)
0	2.58 a[2]
5	2.58 a
10	2.18 a
20	2.9 a

[1]Each value is the mean of 2 replications performed in 3 independent experiments.
[2]Entries within column followed by the same letter are not significantly different at the 95% confidence level by two-way analysis of variance.

Similar reasons prevented any statistical distinction between growth rates of the callus line cultured in hermetic bottles or in Petri dishes. However, development of calli cultured in hermetic vessels seemed generally more heterogeneous.

Silver nitrate did not significantly modify the amount of C_2H_4 included in the gaseous environment of the callus cultures (Table 2). When subcultured in hermetic serum bottles, the calli produced an average of $2.55 \pm 0.42\,\mu l\,l^{-1}$ of C_2H_4 after two weeks of culture.

Plant regeneration

The regeneration capacity was significantly improved when the type II calli were initiated and maintained on a medium complemented with $AgNO_3$

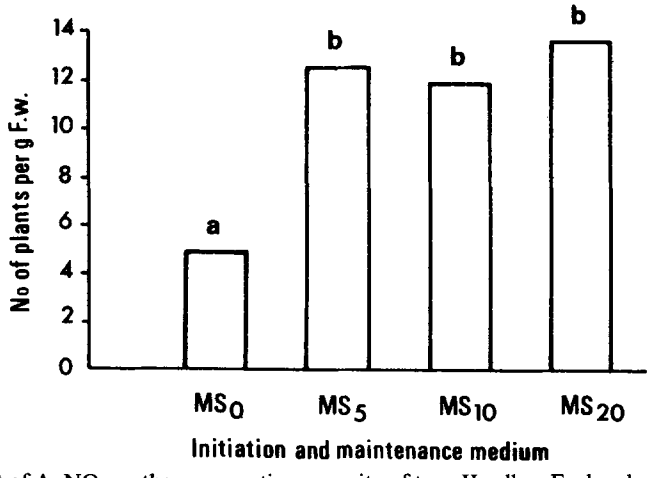


Fig. 5. Effect of $AgNO_3$ on the regeneration capacity of type II callus. Each column represents the number of plants regenerated per g FW. For each medium, 7 type II callus lines have been randomly retained to test their regeneration ability. Entries followed by different letters (a, b) are significantly different at $P = 0.05$ by one-way analysis of variance.

(Fig. 5). Only a mean of 5 plants per g FW of type II callus were obtained with MS_0 against 12 plants per g FW with MS_5, MS_{10} and MS_{20}. Differences for the regeneration ability were maintained at least 8 months after callus initiation (16 subcultures).

Discussion

It has been shown here that silver nitrate enhances the frequency of type II callus initiation without any modification of type I callus production, showing a specific action of $AgNO_3$ on friable tissues during callus initiation process. However, the promoting effect of silver nitrate on regeneration has already been reported in type I callus cultures [20] and therefore doesn't seem specific to type II callus.

The Ag^+ ion is known to be an inhibitor of ethylene action which does not interfere with the C_2H_4 biosynthesis pathway, but with C_2H_4 incorporation at its receptor sites [4]. In our tissue culture experiments the presence of Ag^+ did not modify the production of ethylene in accordance with the results of Le Guyader on tobacco [13].

Various plant cell cultures produce ethylene. Many reports showed a depressive action of C_2H_4 on growth and development of plants in vitro [9, 14] and on regeneration in tissue cultures [10, 18, 20], however, several experiments have led to the opposite conclusion [5]. The main problem for a general understanding is that C_2H_4 acts inside the tissues where it is produced before being released and is efficient at very low concentrations.

This work aims to interpret the positive action of $AgNO_3$ on the initiation of type II callus to counteract ethylene induced by stress [23] during immature embryo extraction, by possible wounding [23] and by the presence of auxins in culture medium [24]. This interpretation will have to be confirmed by the utilization of promotors and inhibitors of ethylene synthesis. Recent work on other maize tissue cultures [20] supports this interpretation for regeneration enhancement.

In our tissue culture conditions, the use of silver nitrate during callus initiation process gave the same initiation rates of type II callus as those obtained with L-proline i.e. roughly 30% [1, 8]. In regard to the results obtained with other basal medium [1, 21], our control medium (MS_0) appeared to be rather favourable for type II callus initiation.

No modification in callus growth has been observed, implying no remarkable effect of silver nitrate on cellular divisions; on the contrary, callus initiation and plant regeneration are influenced by the presence of $AgNO_3$ in the culture medium, indicating that at these particular moments, sensitivity to ethylene might increase.

A genetic capacity exists for several maize genotypes to produce type II callus and regenerate plants after long-term culture [1, 8], but, in order to extend the genetic variability available for tissue culture, conditions need to be improved. Knowledge of the role of C_2H_4 and more generally of the gaseous environment during immature embryo culture might be a way to increase the number of genotypes able to produce type II callus.

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