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Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$

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ABSTRACT

Silver nitrate effectively promoted shoot regeneration in wheat (Triticum aestivum L.) callus cultures derived from immature embryos. This effect could be observed in both weakly and strongly regenerating cultivars, and in using material from both field and greenhouse grown plants. The role of silver ions as an inhibitor of ethylene action was supported by a reversal of the inhibitory effects of 2,4-D and ethylene on morphogenesis in wheat callus cultures.

Enhancement of shoot regeneration by silver nitrate was also observed in callus cultures of non-regenerating or weakly regenerating mutants of Nicotiana plumbaginifolia Viv. derived from cell cultures.

Abbreviations
BA: 6-benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; Ethrel: 2-chlorethylphosphonic acid; NAA: 1-naphthaleneacetic acid; NR^-: nitrate reductase deficient

INTRODUCTION

Shoot regeneration is of crucial importance in the realization of the potential of cell and tissue culture techniques for plant improvement. Callus cultures of the economically important monocots, however, have poor regeneration ability and great differences may exist between genotypes (Sears and Deckard 1982, Duncan et al. 1985). Such genotypic variation in the morphogenic response is widespread, and can even be observed in genera like Nicotiana, widely used as a model in tissue culture studies.

Auxins, which are essential for callus induction, play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration. Auxins can strongly promote the endogenous production of ethylene (see review by Yang and Hoffman 1984). Shoot forming tobacco callus tissues contain significantly lower concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (Grady and Bassham 1982) and produce less ethylene (Huxter et al. 1981) compared to non-shoot-forming callus tissues. It has also been shown that exogenously applied ethylene (or ethylene precursor) in high concentrations inhibited shoot primordium formation in callus cultures (Huxter et al. 1981).

These results suggest that in non-regenerating callus cultures auxin induced ethylene production may be responsible for the suppression of shoot regeneration. We supposed that by inhibition of ethylene action the main effects of auxin (promotion of callus growth and suppression of shoot development) should be separable. In order to promote shoot regeneration we tried to abolish ethylene action by Ag^+ which is known to be a potent inhibitor of ethylene action in plants (Beyer 1976). Ag^+ was tested as $AgNO_3$ on different wheat and Nicotiana genotypes with poor regeneration ability.

MATERIALS AND METHODS

Wheat (varieties GK Maraton and GK Kincső) callus cultures were initiated from immature embryos excised from surface sterilized seeds 11-15 days after anthesis. The embryos (1-1.5 mm) were placed on nutrient agar medium with the scutellum uppermost. For callus induction the culture medium consisted of Murashige and Skoog's (1962) inorganic nutrients, 1 mg/l thiamine-HCl, 100 mg/l meso-inositol, 2 % sucrose, 0,7 % Bacto-Agar and 2,4-D 1.0 mg/l. The cultures were incubated at 25°C (1,000 lx/16 h day). Four weeks after initiation entire pieces of calli were transferred to the above mentioned medium with decreased (0.5 mg/l) 2,4-D concentration. In the third phase (shoot and root regeneration), after a further four weeks, calli were transferred onto MS medium free of 2,4-D, meso-inositol and thiamine-HCl.

Nicotiana plumbaginifolia callus cultures were induced on RMOP medium (Sidorov et al. 1981) from leaf segments of axenic shoot cultures. Non-regenerating N. plumbaginifolia nitrate reductase deficient (NR^-) cell lines (Márton et al. 1985) were maintained on RMOP medium supplemented with 8.25 mM ammonium succinate (Márton et al. 1982). After four weeks the calli formed were cut into 0.5 cm pieces and transferred onto the same medium

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for shoot regeneration. Other conditions were the same as described for wheat cultures.

Hormones and $AgNO_3$ were added to the media prior to autoclaving in a pressure cooker. Ethrel was added to the sterilised medium at 45°C from a filter sterilised stock solution (freshly prepared, pH 5.0). Petri dishes were sealed in each case by commercial cling film.

RESULTS

Preliminary indications that $AgNO_3$ over a concentration range of 5-50 mg/l, can dramatically promote shoot regeneration in wheat callus cultures (Fig. 1) promted a more

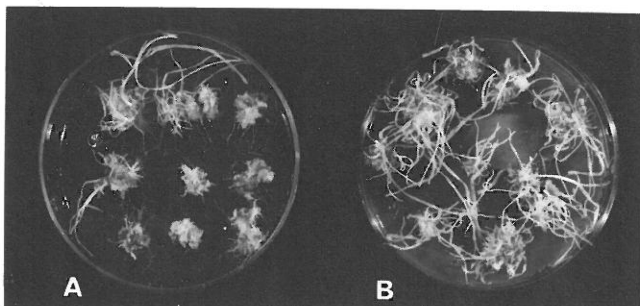

Fig.1. Wheat (GK Kincső) callus cultures derived from immature embryos (from greenhouse plants) on hormone and vitamin free MS medium in the absence (A) or presence (B) of 50 mg/l $AgNO_3$.

detailed study using weakly (GK Maraton) and strongly (GK Kincső) regenerating wheat cultivars. Ten mg/l was chosen for these studies, as higher levels caused necrosis of nonmorphogenic parts of the cultures.

$AgNO_3$ was supplied from the commencement of the first, second or third stages of culture, as defined in the MATERIALS AND METHODS. Clearly the most effective treatment for promoting shoot regeneration in field grown GK Maraton was that in which $AgNO_3$ was present throughout the culture period (Fig. 2). The data presented relate to the number of explants showing shoot regeneration, but there were also increases (usually to about double) in the numbers of shoots appearing on each regenerating explant, resulting in an order of magnitude increase in shoot yield of the culture. Embryos from greenhouse grown plants invariably responded poorly in culture, relative to their field grown counterparts, and in GK Maraton shoot regeneration seldom occurred. $AgNO_3$, applied from the culture initiation stage onwards, however, was again able to dramatically improve the shoot regeneration response (Fig. 4A).

These trends were also observed in cultures of GK Kincső (Fig. 3A and 4B) except that using field grown plants the percentage increase of explants showing regeneration was not significant (Fig. 3A); even a reduction was observed when $AgNO_3$ was applied from the commencement of the first stage of culture (data not shown). This cultivar,

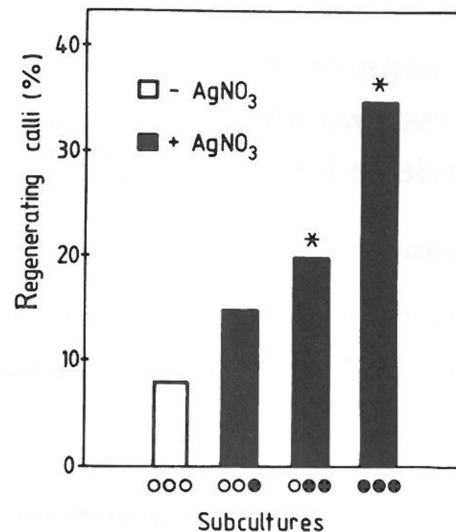

Fig.2. Effect of $AgNO_3$ (10 mg/l) on shoot regeneration in wheat (GK Maraton) callus cultures derived from field grown plants. The media contained $AgNO_3$ in none (OOO), the last (OO●), the last two (O●●) or all (●●●) of the culture steps. An asterisk indicates that the value is significantly (p=0.05) different from the control according to Student's t-test (n=110).

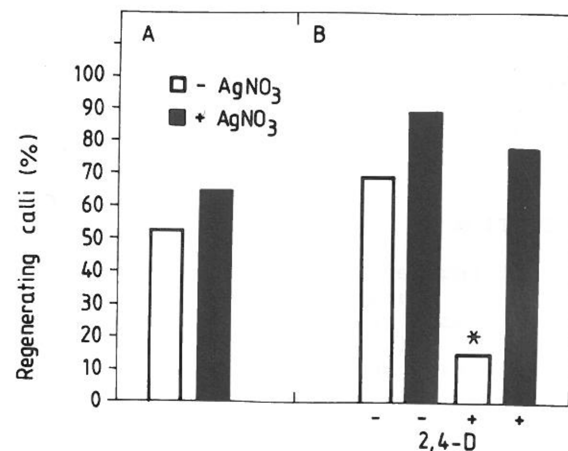

Fig.3. Effect of $AgNO_3$ on shoot regeneration in wheat (GK Kincső) callus cultures derived from field grown plants: (A) with or without addition of $AgNO_3$ (10 mg/l) in the last two culture stages, (n=100) and (B) with or without addition of $AgNO_3$ (10 mg/l) and 2,4-D (0.5 mg/l) in the third culture stage only, and using selected inocula (see text), (n=36). An asterisk indicates that the value is significantly (p=0.05) different from the control (zero 2,4-D and $AgNO_3$) according to Student's t-test.

because of its greater regeneration frequency, was used to investigate the interaction between the synthetic auxin 2,4-D and $AgNO_3$.

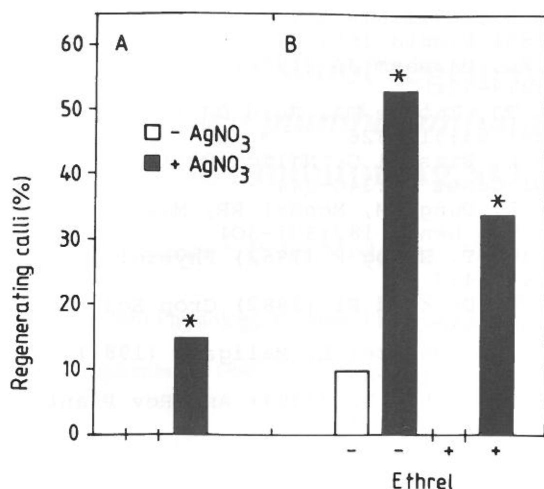

Fig.4. Effect of $AgNO_3$ on shoot regeneration in wheat callus cultures derived from greenhouse grown plants: (A) GK Maraton cultures with or without addition of $AgNO_3$ (10 mg/l) in all three culture stages and (B) GK Kincső with or without addition of $AgNO_3$ (10 mg/l) and ethrel (70 μM) in all three culture stages. An asterisk indicates that the value is significantly (p=0.05) different from the control (zero Ethrel and $AgNO_3$) according to Student's t-test (n=90).

These experiments were performed using only the third culture period, and were restricted to inocula from callus pieces containing compact and yellowish-green parts. This type of tissue generally has a good regeneration ability. This accounts for the elevated frequencies of shoot regeneration, compared to those in which the whole callus is used (Fig. 3A). 2,4-D (at 0.5 mg/l) strongly inhibited shoot regeneration but its effect was circumvented by the presence of $AgNO_3$ in the medium (Fig. 3B).

Other frequently used growth regulators, BA (1 mg/l) and NAA (1 mg/l) did not significantly influence the frequency of shoot regeneration and showed no modification of the $AgNO_3$ effect (data not shown). Using the variety Kincső the effect of the ethylene releasing agent Ethrel (70 μM) applied from the beginning of callus induction was also examined. Ethrel completely suppressed shoot regeneration and its effect could be largely reversed by $AgNO_3$ (Fig. 4B).

Other effects of $AgNO_3$, observed on morphogenic wheat cultures, but for which data are not included, were a general anti-senescence effect (i.e. shoots developing on $AgNO_3$ containing medium remained green and healthy for a longer time than those regenerated on $AgNO_3$ free medium) and a reduction of adventitious root formation (see Fig. 1). The latter effect did not impair recovery of normal plants, however, as satisfactory rooting could subsequently be achieved by transferring the regenerating cultures onto $AgNO_3$ free medium.

In our laboratory a number of nitrate reductase deficient and other cell culture derived mutants (eg. an atypical streptomycin resistant line: SR44) of *Nicotiana plumbaginifolia* show weak or no regeneration ability on the RMOP medium (suitably modified for NR^- mutants) which normally result in prolific shoot regeneration. In all these lines efficient shoot regeneration could be induced by the inclusion of a suitable concentration of $AgNO_3$ in the medium (Table 1).

Table 1. Effect of $AgNO_3$ on the regeneration ability of *Nicotiana plumbaginifolia* callus cultures

Cell lines[a]	Number of calli regenerating/tested	
	$-AgNO_3$	$+AgNO_3$[c]
2/8+NX9(17A)129[a]	1/14	13/14
2/8+NA9(1F)109[a]	0/14	14/14
NX21[a]	0/14	14/14
NA1[a]	0/14	12/14
NA9[a]	1/14	13/14
SR44[b]	0/36	16/36
wild-type	14/14	14/14

[a] Nitrate reductase deficient (NR^-) lines

[b] Streptomycin resistant line

[c] NR^- lines, wild-type: 50 mg/l; SR44: 10mg/l $AgNO_3$

DISCUSSION

This study demonstrates that Ag^+ (a known inhibitor of ethylene action) promotes shoot regeneration in tissue cultures of two wheat cultivars and a number of *N.plumbaginifolia* mutants. Similar observations have been made in several other wheat cultivars, and our preliminary results on plantlet formation in pearl millet (*Pennisetum americanum*) embryogenic callus cultures (the effect on induction and maintenance of embryogenic cultures was not investigated), and runner formation in strawberry (*Fragaria ananassa*) shoot cultures (personal communication of J.Zatykó, Inst. for Fruit and Ornamental Plant Growing, Fertőd, Hungary) imply a more general applicability of $AgNO_3$. These results support a role for ethylene in suppressing morphogenesis in plant tissue cultures. The antagonistic effect of exogenously applied ethylene (as Ethrel) or 2,4-D suggest that the negative influence of 2,4-D on shoot regeneration is mediated through the stimulation of ethylene production.

Beyer (1979) has suggested that Ag^+ interferes with incorporation of ethylene at its receptor site, but not its oxidation to CO_2, in etiolated pea seedlings. A $AgNO_3$ effect on liverwort morphogenesis was interpreted as a desuppression of potential development

of local populations of cells suppressed by ethylene (Basile and Basile 1983). Whatever the precise physiological basis of $AgNO_3$ activity, its practical potential as a tool to optimalize shoot regeneration in cultures with pre-existing but auxin/ethylene suppressed regeneration capacity is clear.

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