J. Hort. Sci.
Vol. 2 (1): 13-18, 2007

Influence of ethylene inhibitors and ethrel on production of protocorm like bodies in orchid - *Dendrobium* 'Sonia'

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ABSTRACT

To increase the efficiency of production of protocorm like bodies, ethylene inhibitors like silver nitrate, salicylic acid and cobalt chloride at three concentrations, viz., 2, 5 and 20 µM and ethrel at 0.7, 1.4 and 14.0 µM were tested, by supplementing MS basal with BAP 4.44 µM. The explant used was fractionated protocorm like bodies (plb). Ethylene and methane gases evolved by the explant in the culture container were measured at 20 and 40 days after inoculation (DAI). Among various ethylene inhibitors tested, the maximum number of plb's were produced in media containing silver nitrate (5 µM), cobalt chloride (2 µM) and salicylic acid (20 µM). All ethrel treatments produced succulent, vitrified and deformed plb's. No ethylene evolution was observed in any of the ethylene inhibitor treatments. Only in ethrel treatments was evolution of ethylene observed. Methane evolution was observed in all the ethylene inhibitor treatments. Absolute amounts of methane evolved could not be related to the observed plb production. However, when the evolution of methane was more than 1 nano mole g^{-1} FW h^{-1}, poor plb production was observed.

Key words: Somatic embryogenesis, methane, silver nitrate, cobalt chloride, salicylic acid

INTRODUCTION

Orchids, one of the largest and important groups of flowering plants, are known for their lovely blooms. They exhibit a great diversity of color, shape, size and shelf-life in their flowers. When there is a need to mass propagate a new orchid hybrid or variety within a short time period, tissue culture is the only method currently available. Among the commercially important orchids, *Dendrobium* accounts for a major share of the total micropropagated tropical orchids (Malabadi *et al*, 2005).

As orchid protocorms are very fragile and need proper and steady nutrient supply for complete differentiation into plantlets, their transportation is usually difficult and expensive. Artificial seeds, also referred as synthetic seeds, are produced by encapsulation of protocorm like bodies in an alginate matrix. This system serves as a low cost, high volume propagation system (Redenbaugh *et al*, 1987). Micropropagation through shoot-tips is successful. However, it is time consuming and laborious. Thus, there is a necessity to develop a sound synthetic seed production (encapsulated plb's) technology applicable for rapid mass propagation and long-term conservation of orchids.

Beneficial effects of ethylene inhibitors on *in vitro* regeneration and somatic embryogenesis were documented in several crops. Ethylene action and biosynthesis inhibitors like silver nitrate increased embryogenesis in carrot (Roustan *et al*, 1990), salicylic acid in *Brassica* (Palmer, 1992) and $CoCl_2$ in carrot (Roustan *et al*, 1989).

There has been increasing evidence that occurrence of morphogenesis and differentiation in cultured plant cells is associated with ethylene (Biddington, 1992). Even there are some indirect evidences which suggest that methane could be involved in the differentiation process (Mustafa *et al*, 1991; Hagege *et al*, 1994). Preliminary studies in our laboratory indicated the possible role of methane in differentiation or regeneration of chick pea, (Chandra *et al*,1997). The above studies in other crops prompted us to examine the role of methane and ethylene on plb's production in orchid – *Dendrobium* 'Sonia'. The objective of the present study was to increase the efficiency of mass production of plb's in orchid – *Dendrobium* 'Sonia' in order to develop sound synthetic seed technology (for rapid propagation). In order to achieve this, effect of various ethylene inhibitors and ethrel (supplemented to MS basal + BAP 4.44 µM medium) on protocorm like bodies production in orchid – *Dendrobium* 'Sonia' was studied.

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MATERIAL AND METHODS

Preparation of fractionated plb explant

Initially shoot-tip explants of orchid – *Dendrobium* 'Sonia' were cultured on MS medium supplemented with BAP 4.44 µM + NAA 5.38 µM for 120 days, which produced several clumps of plb mass (Saiprasad *et al*, 2001). These plb clumps whose apical portion is severed and basal portion was fractioned into 2-3 parts of 0.4-0.5 cms, were used as explants. These explants were inoculated under aseptic conditions to culture tubes containing sterilized nutrient media.

Preparation of filter-sterilized ethylene inhibitors and ethrel

All ethylene inhibitors and ethrel were first dissolved in appropriate solvent or sterile distilled water. Tarsons reusable filter sterilized unit containing 47 mm diameter 0.2 mm bacterial filter membrane was first autoclaved as described below and was taken inside laminar air flow unit. The aqueous solution was slowly filtered and was stored in well stoppered glass bottles inside refrigerator (0°C) and used depending on the requirement.

Culture tubes and sterilization of nutrient media

Murashige and Skoog (1962) basal medium (containing 0.8% agar, 3% sucrose) supplemented with BAP 4.44 µM and pH adjusted to 7.5 ± 0.1 was autoclaved at 15 lb/psi for 15 min. at 121°C and was allowed to cool down to 50-60°C, then previously filter sterilized chemicals (ethylene inhibitors and ethrel) were added as per the required concentration in the laminar air flow unit. This was then poured into previously autoclaved culture tubes (150mm x 25mm). About 20 ml of medium was poured into each culture tube.

Effect of ethylene inhibitors and ethrel

Ethylene inhibitors, silver nitrate, salicylic acid and cobalt chloride, all at 3 concentrations each (final) viz., 2, 5 and 20 µM and ethrel at 0.7, 1.4 and 14.0 µM supplemented to Murashige and Skoog (1962) medium with BAP 4.44 µM medium (control) were tested.Influence of these ethylene inhibitors and ethrel on plb's production as a function of ethylene/methane evolved was studied.

Culture conditions

The cultures were incubated at a temperature of 25 ± 2°C, with a 16 h light and 8 h dark photoperiod (Irradiance of 50-60 mmE m^{-2} S^{-1}) for about 6 weeks.

Measurement of ethylene/methane

Ethylene/methane were measured at 20 and 40 days after inoculation (DAI). Cotton plugs of culture tubes were replaced by airtight serum caps and were incubated for 24 h before taking observations. Preliminary experiments conducted showed a virtual linear relationship between inoculation time and ethylene/methane evolution up to 24 h. Hence, ethylene and methane were measured always for 24 h after incubation.

For each assay, 2 ml of gas samples were with drawn from each tube and were injected into gas chromatogram (*Sigma 2000, Perkin-Elmer*, USA) equipped with *Porapak N 80/100* mesh column and a flame ionization detector (FID). Carrier gas (nitrogen) flow was maintained at 20 cm^3 min^{-1}. Column temperature was maintained at 60°C and that of injector and detector at 200°C. Standard ethylene (105 vpm) and methane (108 vpm) obtained from EDT Research, London were used for calibration and injected in a similar way as samples.

Data recorded

Number, fresh weight (FW) and dry weight of plb's and amount of ethylene or methane evolved in n mol per g^{-1} FW h^{-1} were recorded at 20 and 40 DAI. Three culture tubes were used per treatment with single explant for measuring ethylene/methane production along with data on fresh and dry weight and six replications were used for evaluating the number of plb's.

The data were recorded at both 20 and 40 DAI and were analyzed with completely randomized design by following standard methods (Gomez and Gomez, 1984) using microstat software developed by Barreto, HJ, Raun, WR; CIMMYT, Mexico. Means were evaluated at p=0.05 level of significance using Duncan's new multiple range test. Each experiment was repeated twice.

RESULTS AND DISCUSSION

Effect of ethylene inhibitors and ethrel

(a) Plb's production

Maximum production of plb's was recorded in $AgNO_3$ (5 µM), followed by $CoCl_2$ (2 µM) and salicylic acid (20 µM) treatments at both 20 and 40 DAI (Table 1 and 2). Decreased production of plb's was observed when concentration was either increased or decreased beyond the optimum level cited for the respective ethylene inhibitors (Plate. 1a, 1b and 1c). All ethrel treatments have only resulted in production of succulent, vitrified and deformed structures, except 1-2 plb's at 0.7 µM ethrel treatment. At higher concentration of ethrel treatments browning and drying of tissue was observed (Plate 1d).

(b) Fresh weight and dry weight

Maximum fresh weight was recorded in $AgNO_3$ (5 µM), followed by $CoCl_2$ (2 µM) treatments at both 20 and 40 DAI. $AgNO_3$ (2 µM), salicylic acid (5 µM) and all ethrel treatments recorded significantly lower fresh weight than control. Dry weight observations recorded similar pattern as that of fresh weight, at both 20 and 40 DAI measurements.

(c) Methane and Ethylene

Significant differences in methane evolution were observed among the different ethylene inhibitors and ethrel treatments both at 20 and 40 DAI (Table 1 and 2). In all the treatments the rate of methane evolved at 20 DAI was much higher than that evolved at 40 DAI. The absolute amounts (rate) of methane evolved was not related to the plb's production response at both 20 and 40 DAI. In general, at 40 DAI when the evolution of methane is more than 1.0 nano mole per gram FW h^{-1} ($AgNO_3$ 2 µM and all ethrel treatments) succulent, vitrified and deformed structures other than plb's were produced (Plate 1). These tissues latter turned brown and died. Ethylene production was observed in all ethrel treatments only, but could not be detected in ethylene inhibitor treatments. The amount of ethylene evolved was observed to increase with the increase in concentration of ethrel at both 20 and 40 DAI (Table 1 and 2).

Table 1: Effect of various ethylene inhibitors and ethrel on production of plb's, fresh weight, dry weight, methane and ethylene at 20 DAI

Ethylene inhibitors	No. of plb's/ culture tube	Fresh weight (mg)	Dry weight (mg)	n moles g^{-1} FW h^{-1}	
				Methane	Ethylene
Silver nitrate 2 µM	5.17 (2.38)e	92.05ef	10.03de	1.347g	ND
Silver nitrate 5 µM	26.17 (5.15)a	167.30a	14.71a	0.999g	ND
Silver nitrate20 µM	13.17 (3.69)c	135.50bc	12.65abc	1.315g	ND
Cobalt chloride 2 µM	21.50 (4.68)b	158.06ab	14.53ab	2.307ef	ND
Cobalt chloride 5 µM	11.83 (3.50)cd	123.00cd	11.68cd	2.758de	ND
Cobalt chloride 20 µM	9.83 (3.19)d	131.75c	12.45abcd	1.253g	ND
Salicylic acid 2 µM	6.67 (2.67)e	115.07cde	12.16bcd	5.810a	ND
Salicylic acid 5 µM	6.67 (2.67)e	112.08cde	10.93cde	4.473b	ND
Salicylic acid 20 µM	12.00 (3.52)cd	106.53de	10.77cde	1.908f	ND
Ethrel 0.7 µM	1.50 (1.40)f	80.03fg	9.17ef	2.100f	0.158b
Ethrel 1.4 µM	0.00 (0.71)g	78.06fg	8.95ef	3.254cd	0.161b
Ethrel 14 µM	0.00 (0.71)g	67.33g	7.57f	3.610c	1.654a
MS basal + BAP 4.44 µM (control)	13.67 (3.75)c	156.47ab	14.25ab	2.380ef	ND
C.D. (*P=0.05*)	0.323	24.337	2.402	0.559	0.174

Means followed by common letters within a column are non-significant at 5%
Figures in parentheses indicate transformed values; ND : Not detected

Table 2. Effect of various ethylene inhibitors and ethrel on production of plb's, fresh weight, dry weight, methane and ethylene at 40 DAI

Ethylene inhibitors	No. of plb's/ culture tube	Fresh weight (mg)	Dry weight (mg)	n moles g^{-1} FW h^{-1}	
				Methane	Ethylene
Silver nitrate 2 µM	9.33 (3.13)e	207.00e	16.03e	1.055bc	ND
Silver nitrate 5 µM	44.17 (6.65)a	854.69a	67.54a	0.364g	ND
Silver nitrate20 µM	32.17 (5.70)b	616.86bc	48.42bc	0.521efg	ND
Cobalt chloride 2 µM	40.50 (6.39)a	700.02b	54.54b	0.418fg	ND
Cobalt chloride 5 µM	26.50 (5.19)cd	504.84cd	38.36cd	0.375g	ND
Cobalt chloride 20 µM	22.67 (4.80)d	634.03bc	49.93b	0.349g	ND
Salicylic acid 2 µM	11.50 (3.46)e	630.02bc	49.49b	0.646def	ND
Salicylic acid 5 µM	10.00 (3.23)e	458.05d	36.10d	0.730de	ND
Salicylic acid 20 µM	28.50 (5.36)bc	587.21bcd	44.35bcd	0.874cd	ND
Ethrel 0.7 µM	2.17 (1.56)f	136.78e	14.36e	1.183b	0.085c
Ethrel 1.4 µM	0.00 (0.71)g	103.22e	11.97e	1.287b	0.199b
Ethrel 14 µM	0.00 (0.71)g	80.91e	9.85e	1.518a	0.265a
MS basal + BAP 4.44 µM (control)	25.67 (5.07) cd	597.83 bc	45.93bcd	0.680de	ND
C.D. . (*P=0.05*)	0.443	138.02	10.145	0.241	0.028

Means followed by common letters within a column are non-significant at 5%
Figures in parentheses indicate transformed values. ND : Not detected

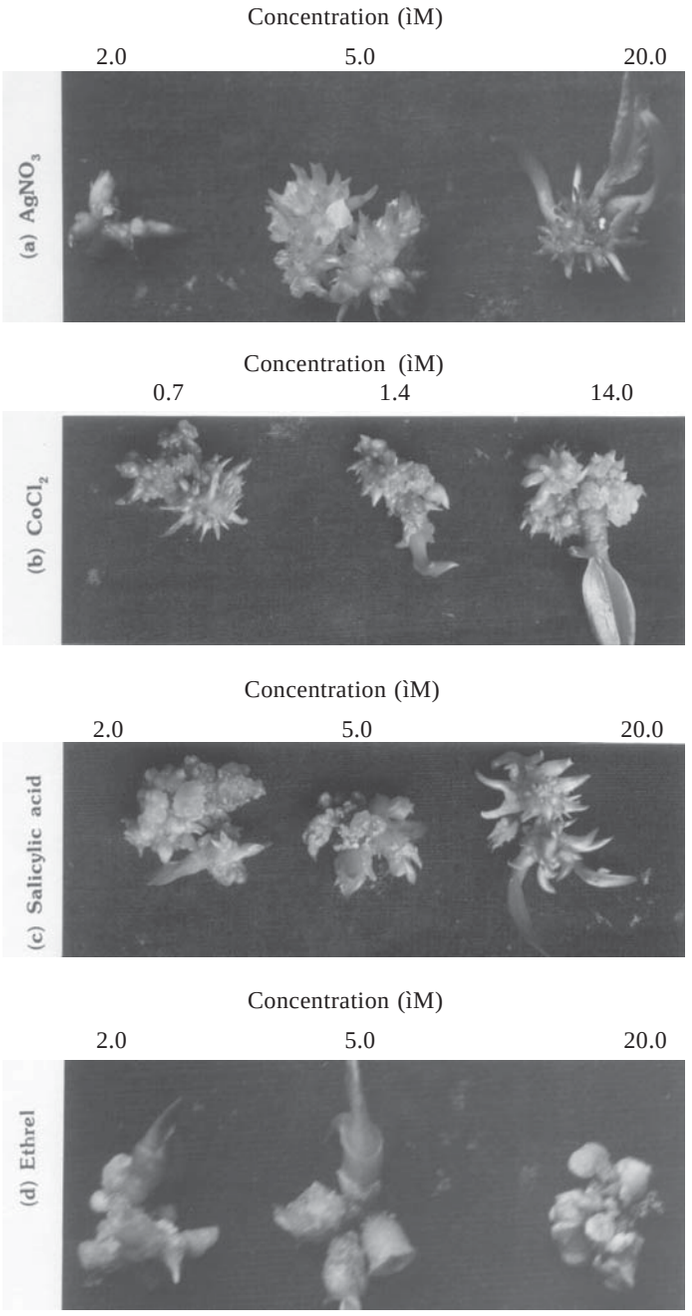

Fig 1. Effect of various ethylene inhibitors and ethrel supplements to MS basal +BAP 4.44 µM on production of plb's/multiple shoots by frctionated plb explants of Dendrobium sonia at 40 DAI

Ethylene produced by plant tissue (explant)/tissue grown *in vitro* may accumulate in large quantities in the culture vessels and hence, likely to influence the growth and development in such system. There are several reports in literature which support that ethylene influences callus growth, shoot regeneration and somatic embryogenesis *in vitro* (Biddington, 1992; Sankhla *et al*, 1995). Surprisingly no ethylene could be detected in the control treatment. Such a result would suggest either ethylene has no role in plbs production or no detectable ethylene evolution occurred as a result of interaction between explant and MS medium supplemented with BAP (4.44 µM). However when ethylene was supplemented to the media in the form of ethrel it resulted in significant levels of ethylene evolution. In these ethrel treatments plbs production was arrested and plb's formed were deformed, succulent and vitrified and even these tissues turned brown within next 20-30 days. Thus, this clearly indicates that, ethylene adversely effects plb/multiple shoot formation. Ethylene in the culture medium supplemented to control treatment, however, did not prevent methane accumulation. In fact methane evolution at 40 DAI measurement was significantly higher (more than 100% increase) than control treatment.

The efficiency of plb production varied with the ethylene inhibitors tested. Most ethylene inhibitor treatments significantly increased the plb production. Number of protocorm like bodies produced was observed to be dependant on the specific ethylene inhibitor and concentration of inhibitor used. For instance, enhanced plb production was observed only in 5 µM $AgNO_3$ (% increase 72.07), 2 µM $CoCl_2$ (% increase 57.77) followed by 20 µM $AgNO_3$ (% increase 25.32) at 40 DAI. It is interesting to note that other ethylene inhibitor treatments recorded either significantly lower plb production or on par with control. In these treatments no significant amount of ethylene was detected and reasons for the specific concentration response is not known.

Blocking the conversion of ACC to ethylene by ethylene inhibitors ($AgNO_3$, $CoCl_2$ and salicylic acid) inhibiting the ACC oxidase was studied extensively by several workers. Addition of $AgNO_3$ promoted shoot bud regeneration in wheat and tobacco (Purnhauser *et al*, 1987), in *Brassica* (Palmer, 1992), in rice (Adkins *et al*, 1993), in silk tree (Sankhla *et al*, 1995), in muskmelon (Yadav *et al*, 1996) and in potato (Zobayed *et al*, 2001; Naik *et al*, 2003).

Inhibition of ethylene production by $CoCl_2$ resulting in increased shoot regeneration was reported, for instance in *Brassica oleracea* (Sethi *et al*, 1990); in *Brassica campestris* (Palmer, 1992); in pearl millet (Pius *et al*, 1993) and in silk tree (Sankhla *et al*, 1995). Inhibition of ethylene production by salicylic acid was reported in carrot (Roustan *et al*, 1992) and in *Brassica campestris* (Palmer, 1992).

Critical evaluation of ethylene inhibitor and ethrel treatments indicates presence of methane at varying concentrations. In all the treatments, ethylene evolution was suppressed. Methane level higher than 1 n mol g^{-1} FW h^{-1} at

40 DAI has inhibited plb development and promoted either callus or vitrified deformed plb production. This study raises a few questions, i.e., what is the source (primary and secondary substrate) of methane production in ethylene inhibitor treatments? Absence of ethylene in these treatments suggests that in the orchid system production of ACC particularly from S-adenosyl methionine could be severely inhibited. It is speculated that ACC synthase, may be absent or inactive leading to negligible synthesis of ACC. However, it has to be experimentally verified. The fact that ethylene action/biosynthesis inhibitors particularly $AgNO_3$ and $CoCl_2$ has enhanced effect on plb's production, which also supports ACC is not formed in the orchid system.

It has also to be experimentally verified, (i) what methane concentration would be beneficial for plb's production/multiple shoot production and (ii) what methane concentration contributes to callus/vitrified or deformed plb's production. (iii) why only methane was evolved at the exclusion of ethylene in this orchid system.

The efficiency of production of protocorm like bodies can be increased by supplementing ethylene inhibitors like $AgNO_3$ (5 µM) or $CoCl_2$ (2 µM) to MS basal + BAP 4.44 µM medium. However, poor plb's production in ethrel treatments indicated that ethylene in the culture medium could adversely affect plb's production in this orchid.

ACKNOWLEDGEMENT

The senor author thanks all the members of the plant tissue culture laboratory in the division of plant physiology at Indian Agricultural Research Institute (IARI), New Delhi, for their constant support and help in carrying out this work. He also acknowledges Indian Council of Agricultural Research (ICAR) for providing financial assistance in the form of Senior Research Fellowship (SRF) for carrying out his doctoral work at IARI, New Delhi.

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(MS Received 15 September 2006, Revised 4 June 2007)