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Effect of Novel Branching Regulators on Micropropagation of *Zantedeschia sprengeri* (Paco)

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THE NOVEL branching regulators fluridone and imazalil (IMA) were examined for micropropagation of Paco (*Zantedeschia sprengeri*). IMA concentrations were 0, 4.5, 13.5 and 45µM in combination with 5µM benzylaminopurine (BAP). The shoots were transferred after 8 weeks to standard MS medium without IMA but with 5µM BAP. IMA resulted in a strong increase of new shoots from which the outgrowth was inhibited when IMA was present but that did grow out when IMA had been removed. Fluridone inhibits the biosynthesis of the endogenous regulator strigolactone. Increasing concentrations of fluridone were applied in combination with 13.5µM IMA for 8 weeks. Then the shoots were subcultured on standard medium without IMA and fluridone but with 5µM BAP for 8 weeks. Again a strong increase of new shoots occurred from which outgrowth was inhibited when IMA and fluridone were present but that did grow out when these regulators had been removed.

Keywords: Apical dominance, Fluridone, Imazalil, Strigolactone, *Zantedechia*.

Introduction

The genus *Zantedeschia*, for the most part known as the arum or calla lily, is bound to the African mainland. Aside from the more regularly known white *Zantedeschia aethiopica* there are six other *Zantedeschia* species and three subspecies with bloom colors running from pink, cream and yellow to ruddy purple. As cut blooms they are exceptionally all around enjoyed everywhere throughout the world and a huge number of cultivars and hybrids are accessible (Tjia, 1989; Kuehny, 2000). In nature, *Zantedeschia* develops in sloppy living spaces that give a decent atmosphere to be tainted infected with rotting bacteria and fungi. From the bulb producer's perspective, an ideal framework for tuber generation of calla lily should pressure extensive tuber size and negligible misfortune because of contamination by *Erwinia carotovoras* pp. carotovora or *E. chrysanthemum* (Welsh & Clemens, 1992).

Financially, multiplication of *Zantedeschia* through counterbalances and tuber division is rehearsed to build the quantity of tubers of blooming size. In any case, divisions of field-developed tubers are liable to disease by *Erwinia* soft rot, which causes serious losses (Ni et al., 2010). Tissue culture procedures have been created as an option for the generation of tubers in *Z. aethiopica* (Cohen, 1981; Clemens & Welsh, 1993; Fang et al., 1999). Internal contaminants is the most concerning issue and the utilization of rhizomes as the source of explant material makes it hard to set up without contaminant material in vitro (Ruiz et al., 1996; Kritzinger et al., 1998).

In general, vegetative multiplication *in vitro* is achieved by adventitious regeneration of new shoot meristems from somatic tissues or by axillary branching, i.e., outgrowth of axillary meristems. Most micropropagation protocols use the latter pathway. Obviously, plant growth regulators that influence the outgrowth of axillary

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buds are of unequivocal importance. Auxins inhibit and cytokinins promote the outgrowth, but other regulators may also have an effect. In the present study on *Zantedeschia*, we examined IMA that has been reported to promote side shoot formation in *Spathiphyllum* (Werbrouck et al., 1995) and fluridone that blocks the synthesis of strigolactone, a recently identified endogenous inhibitor of the outgrowth of axillary buds (Ferguson & Beveridge, 2009).

Materials and Methods

Plant material

Established *in vitro* cultures of *Zantedeschia* (Paco) were provided by Sande Breeding BV, `t Zand, The Netherlands.

Culture media and culturing of explants

The basic medium was composed of macro- and micronutrients and vitamins according to Murashige & Skoog (1962), 100mg L^{-1} inositol, 3 % sucrose and 2 g l^{-1} gelrite. This basic medium was supplemented with increasing IMA and fluridone concentrations and 5µM BAP. The pH of media was adjusted to 5.7 with 1N KOH and 1N HCl before adding gelrite (0.2%) and prior to autoclaving at 121˚C (0.1 MPa) for 20min. Filter-sterilized IMA and fluridone were added after autoclaving. The cultures were kept in growth chambers at 21˚C± 1˚C, under a photoperiod of 16 hrs (30µ$Em^{-2}s^{-1}$, Philips TL 33). Five jars and five explants/ jar were cultured for each concentration of fluridone.

The effect of IMA

The explants were inoculated upright with the basal plate tissue inserted into the media. The basal media were supplemented with 5µM BAP. Increasing concentrations of IMA (0, 4.5, 13.5 and 45µM) were added using filter sterilization in addition to 5µM BAP after autoclaving. The second part of the experiment was carried out after 8 weeks. The *in vitro* shoots were transferred to MS basal media with 5µM BAP. The number of new shoots was recorded after 8 weeks of culture on different IMA concentrations and after the second 8-w period on medium without IMA. The experiment was repeated three times.

The effect of IMA and fluridone

The explants were cultured aseptically upright with the basal plate tissue inserted into the MS culture media. The basal MS media with 5µM BAP were supplemented with increasing concentrations of fluridone (0, 0.3 and 0.9µM) in addition to 13.5µM IMA. Fluridone was added using filter-sterilization after autoclaving.

The second part of the experiment was carried out after 8 weeks. The shoots were transferred to MS media supplemented with 5µM BAP but without IMA and fluridone and cultured for an additional period of 8 weeks.

The number of new shoots was recorded after 8 weeks of culture on different fluridone concentrations and after the second 8-w period on medium without IMA and fluridone. The experiment was repeated three times.

Statistical analysis

Data were analyzed using the Student *t*-test to compare the effect of different growth regulators on the number of shoots and shoot length. Results were statistically analyzed by a factorial analysis of variance, in completely randomized design according to the procedure by Snedecor & Cochran (1981) and means were compared by multiple range tests.

Results and Discussion

The effect of IMA

Shoots were routinely propagated on Murashige & Skoog (1962) (MS) medium to which 5µM BAP had been added. To examine the effect of IMA, shoots were cultured on MS media supplemented with increasing concentrations of IMA (0, 4.5, 13.5 and 45µM) for a period of 8 weeks. The shoots were then transferred to standard medium containing 5µM BAP and cultured for a second period of 8 weeks. At the end of both periods, the numbers of shoots were scored.

During the first period, 4.5µM IMA resulted in a small increase of the total number of shoots (Fig. 1 B1) ($P< 0.05$). The highest IMA concentration tested (45µM) yielded less shoots than the optimal at 4.5µM (Fig. 2). When the shoots were cultured after the first period with IMA on medium devoid of IMA but with 5µM BAP, the number of shoots was the same with the notable exception of the initial period with 45*µ*M: in this case, the number of shoots during the second period increased to 7.8 (Table 1). The shoots seemed to be of good quality (Fig. 1 D2).

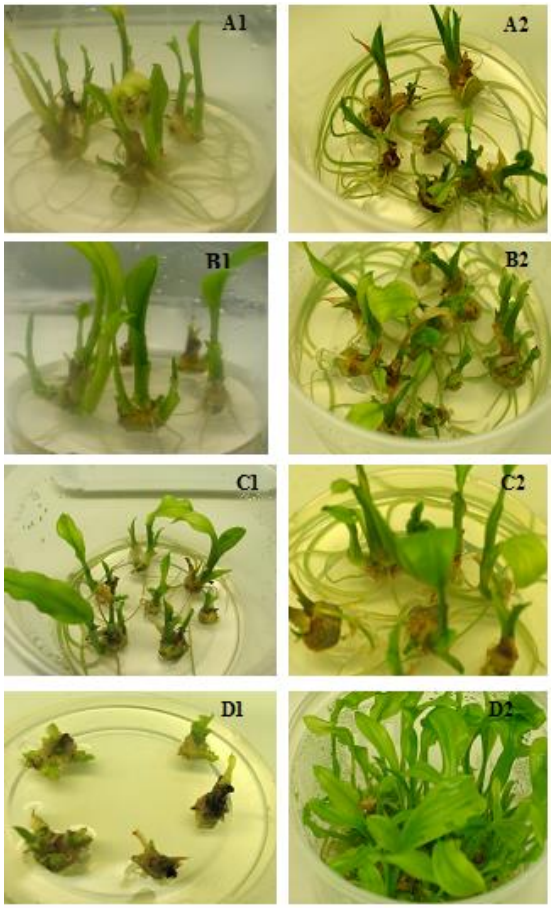

Fig. 1. ***In vitro*** **shoots of** ***Zantedeschia*** **"Paco" after 8 weeks of culture on increasing concentrations of IMA, (A1) control medium with 5µM BAP, (B1) 4.5µM IMA, (C1) 13.5µM IMA and (D1) 45µM IMA [After 8 weeks of culture the shoots were transferred to medium supplemented with only 5µM BAP and after an additional period of 8 weeks photos were taken (A2-D2)].**

TABLE 1. ***In vitro*** **shoots of** ***Zantedeschia*** **"Paco" after 8 weeks of culture on increasing concentrations of IMA shoots were counted and measured, then, the shoots were transferred to medium supplemented with only 5µM BAP and after an additional period of 8 weeks photos shoots were counted and measured.**

IMA conc. µM	Initial Treatment		On standard medium after 8 weeks	
	Shoot No.	Shoot length (cm)	Shoot No.	Shoot length (cm)
No IMA	3.02±0.24	3.40±0.16	2.70± 0.32	4.20± 0.14
4.5	4.21±0.22	2.90±0.11	2.90 ± 0.46	4.50± 0.04
13.5	4.00±0.19	5.20±0.55	3.00± 0.27	5.60 ±0.08
45	3.35±0.21	1.70±0.32	7.80± 0.21	6.30± 0.19
S.D. at 5% level=	1.002	1.264	0.6578	1.027

IMA is an imidazole fungicide. It shares structural features common to this family of compounds, and their target enzymes are monooxygenases (Rademacher, 1991) so they might affect several biochemical pathways. A synergistic action of IMA and BAP on adventitious bud formation has been recently observed by Werbrouck & Debergh (1995) in micropropagated *Spathiphyllum* plants that resembled some of our observations in *Zantesdeschia*.

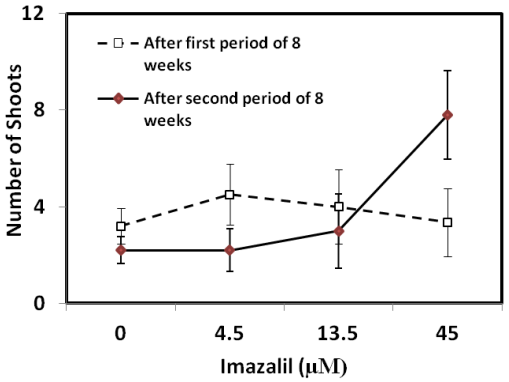

Fig. 2. ***In vitro*** **shoots of** ***Zantedeschia*** **"Paco" after 8 weeks of culture on increasing concentrations of IMA [Shoots were counted, then the shoots were transferred to medium supplemented with only 5µM BAP and after an additional period of 8 weeks photos shoots were counted, each value is the mean of ca 30 explants ±SE].**

The mode of action of IMA is not well understood. It only induces shoots in the presence of exogenous cytokinins such as BAP. This fact indicates that IMA causes some alteration of BAP metabolism. When cultured on a medium containing BAP, *Spathipyllum* mainly converts BAP into large amounts of [9G] BAP, which is considered a storage or a detoxification product and into [9R] BA, which still has cytokinin activity (Werbrouck et al., 1995). IMA might influence the concentration of the active BAP metabolites and could also have an inhibitory effect on their catabolic enzymes. On the other hand, an effect of IMA on GA metabolism cannot be excluded, because of the similar structures of IMA and growth retardants (Piqureas et al., 1999). IMA (Werbrouck & Debergh, 1995) is not the only imidazole fungicide which can considerably enhance the shoot-inducing effect of BA; related fungicides such as prochloraz and triflumizole as well as the growth retardant paclobutrazol, have comparable effects. This synergistic effect was confirmed for such widely different cytokinins as zeatin, meta-topolin, and thidiazuron in combination with IMA. The interaction with this wide selection of cytokinins indicates that IMA probably affects a general mechanism of cytokinin action.

Effect of IMA and fluridone

Shoots were routinely propagated on Murashige & Skoog (1962) (MS) medium to which 5µM BAP had been added. Then the shoots were cultured on MS media supplemented with different concentrations of fluridone in combination with 13.5µM IMA (0, no flur, 0.3 and 0.9µM) for 8 weeks. The shoots were then transferred to standard medium containing 5µM BAP without IMA and fluridone and cultured for a second period of 8 weeks. At the end of both periods, the numbers of shoots were scored.

During the first period, a small effect of fluridone occurred. During the second period, the culture of IMA- and fluridone-treated shoots in the absence of these growth regulators (but with 5µM BAP), there was a strong effect (Table 2). The highest shoot multiplication rate, 10.48, was obtained in shoots grown on culture medium with 0.9µM and 13.5µM IMA (Figs. 3 and 4).

Fluridone likely acted by inhibiting strigolactone synthesis. The two most important hypotheses about the role of strigolactone in shoot branching are the auxin canalization model (Bennett & Leyser, 2006; Leyser, 2009) and the second messenger model (Brewer et al., 2009). The canalization model states that auxin transport canalization from the axillary buds to the stem is necessary for bud outgrowth and that auxin in the main stem, along with strigolactone, influences auxin transport to inhibit bud outgrowth. According to the second messenger model, auxin regulates the production of strigolactones, which are transported to the axillary buds to inhibit outgrowth through the involvement of a bud-specific transcription factor BRC1 (Domagalska & Leyser, 2011). These models are not mutually exclusive as there is experimental evidence in support of both the canalization model (Crawford et al., 2010; Shinohara et al., 2013) and the second messenger model (Brewer et al., 2009; Brewer et al., 2015; Dun et al., 2013).

Conclusion

In the present experiment, we did not find an effect or only a small effect of fluridone when this regulator was still present in the medium but it had a major effect in the following period when both fluridone and IMA were absent (but BAP was added at 5µM). We hypothesize, just as in the case when only IMA was added, that the regulator has its releasing effect but at the same time inhibits outgrowth so that the number of extra shoots is limited.

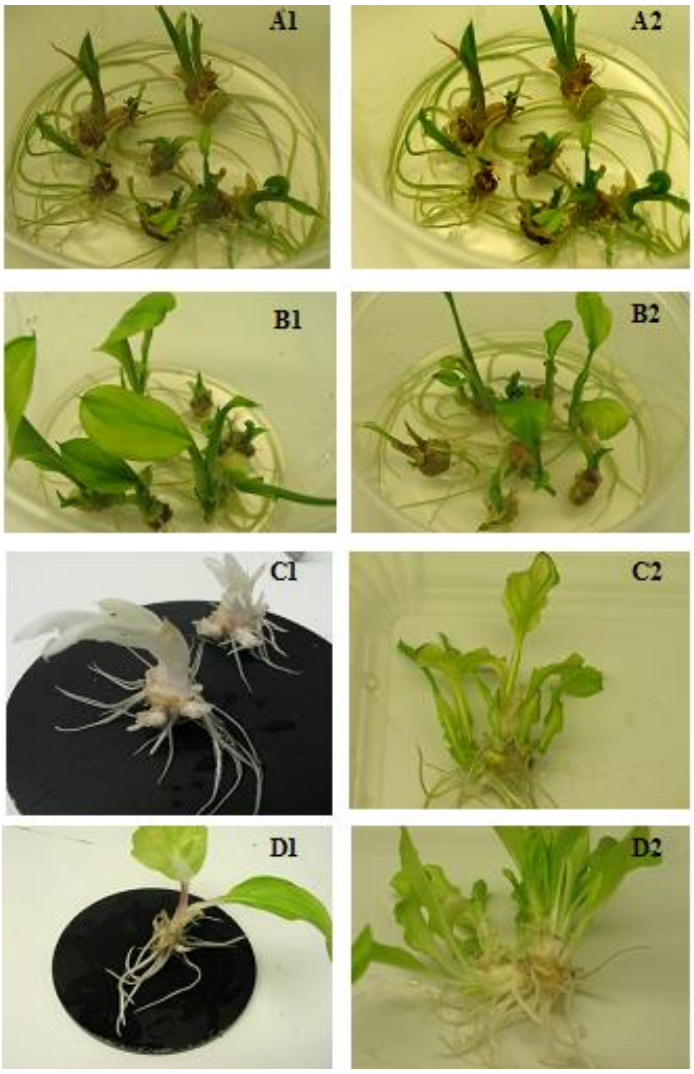

Fig. 3. Increasing concentrations of fluridone were applied to *Zantedeschia* shoots in combination with 13.5µM IMA for 8 weeks and photos were made: (A1) control, (B1) No fluridone, (C1) 0.3µM fluridone, (D1) 0.9µM fluridone [Then they were subcultured on standard medium without fluridone and IMA for 8 weeks and photos were made (A2-D2)].

TABLE 2. Increasing concentrations of fluridone were applied to *Zantedeschia* shoots in combination with 13.5µM IMA. After 8 weeks the shoots were counted and measured, then they were subcultured on standard medium without fluridone and IMA for 8 weeks and again counted and measured.

Fluridon conc. (µM)	Initial treatment		On standard media after 8 weeks	
	Shoot No.	Shoot length (cm)	Shoot No.	Shoot length (cm)
Cont.	3.02± 0.08	3.4±0.18	2.40± 0.19	4.2±0.11
13.5µM IMA (no Fluridon)	4.21± 0.12	2.9± 0.26	2.90 ± 0.12	4.5±0.1
0.3	3.98± 0.22	5.2±0.23	5.80±0.15	5.6± 0.32
0.9	4.47± 0.17	1.7± 0.04	10.48± 0.18	6.3±0.43
L.S.D. at 5% level =	1.010	1.434	0.8235	1.189

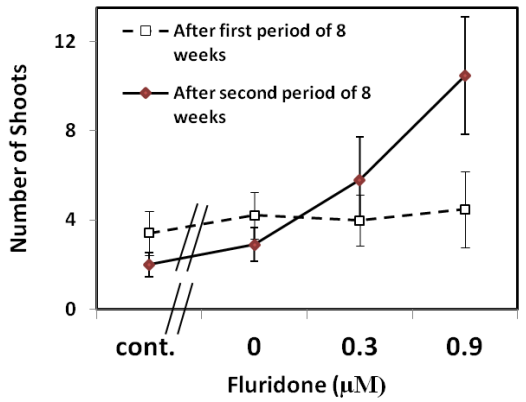

Fig. 4. Increasing concentrations of fluridone were applied to *Zantedeschia* shoots in combination with 13.5µM IMA [After 8 weeks the shoots were counted, then they were subcultured on standard medium without fluridone and IMA for 8 weeks and again counted. Each value is the mean of ca 30 explants ±SE].

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