* VEGETATIVE PROPAGATION OF ALSTROEMERIA HYBRIDS IN VITRO

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Abstract

Terminal and lateral tips from fleshy rhizomes of A l s t r o e m e r i a hybrids were isolated in vitro and induced to form a new rhizome. The cultivar Toledo was used in most experiments, but later other cultivars were also tested. The basic culture medium for rhizome isolation and for rhizome multiplication was: Murashige and Skoog (MS) macro- and micro-salts at full strength (except Fe), NaFeEDTA 25 mg/l, saccharose 3%, BA 2-4 mg/l vitamin B_1 0.4 mg/l, and Difco Bacto-agar 0.7 %. The basic culture medium for rooting was slightly different: saccharose 5%, BA was omitted and 0.5 mg/l NAA was added.

Rhizome cultures were placed at 21°C and 8 h fluorescent light/16 h darkness. Rooting was carried out at 21°C and 16 h fluorescent light/8 h darkness.

Rhizome multiplication required a cytokinin in the medium; BA and PBA were most effective, whereas kinetin, 2iP, and zeatin were not very effective. BA at 2-4 mg/l partially suppressed erect shoot growth and stimulated rhizome branching. Addition of auxin had no effect on rhizome multiplication.

Relative small rhizome explants (with one bud) had a higher multiplication rate than large ones. Optimal rhizome multiplication required 3 week cycles of subculturing; cycles of 4, 5 and 6 weeks being less productive. The multiplication rate was increased by growing the rhizomes in liquid media; however, this resulted in vitrification.

Excised rhizome explants can be rooted by subculturing rhizome explants on cytokinin-free media containing auxin. Generally NAA (optimum 0.5 mg/l) induced better rooting than IBA. In vitro rooted plants were successfully transferred to the greenhouse and developed into normal flowering plants.

1. Introduction

From 1984-1986 the production area of A l s t r o e m e r i a increased worldwide from 200 to 275 ha, and at the same time plant breeding provided many new cultivars of this important cut flower (Anonymous, 1987a, 1987b). The increased interest in A l s t r o e m e r i a is due particularly to its low energy requirement for cultivation and the good keeping quality of its flowers (Hakkaart and Versluijs, 1985; Lin and Monette, 1987). The increase in production area and the introduction of new hybrids by breeding have made necessary to develop efficient methods for cloning since A l-

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s t r o e m e r i a can only be propagated vegetatively since the hybrids are often sterile triploids.

The natural propagation rate of A l s t r o e m e r i a hybrids is rather slow and therefore micropropagation methods, in particular rhizome multiplication, have been developed (Hussey et al., 1979; Gabryszewska and Hempel, 1985; Lin and Manette, 1987); micropropagation was also developed as a means of becoming independent of the seasons. Another reason for cloning A l s t r o e m e r i a hybrids in vitro was to speed up propagation of virus-free plantlets obtained by meristem culture (Hakkaart and Versluijs, 1985).

The main motive behind basic research on A l s t r o e m e r i a micropropagation has been the large production area of this cut flower (62 ha under glass in 1986) in the Netherlands, resulting in auction sales of approximately 125 million flowers in 1986.

2. Materials and methods

Rhizomes of A l s t r o e m e r i a 'Toledo' and other hybrids were selected from greenhouse-grown plants. Prior to sterilization, rhizomes were washed under tap water to remove soil, etc. Surface sterilization of rhizome segments was as follows: 2-3 seconds in alcohol 70%, 20 min in 1.5% NaClO (with a few drops of Tween 20), and then rinsed 3 times (for 5, 10, and 20 min respectively) in sterile tap water. Apical and axillary rhizome tips were subsequently excised under a binocular microscope and resterilized as follows: 10 min in 0.05% NaClO, and rinsing as described above. Rhizome tips were subsequently placed on solid media.

In most experiments rhizome segments were grown on the following basic culture medium: Murashige and Skoog (1962) macro- and micro-salts (except Fe) at full strength, NaFeEDTA 25 mg/l, saccharose 3%, BA 2-4 mg/l (for other cultivars than 'Toledo' see section 3.1.4), vitamin B_1 0.4 mg/l, Difco Bacto-agar 0.7%, and distilled water. The pH was adjusted to 6.0 before autoclaving. In media for adventitious rooting NAA (0.5 mg/l) was added, BA was omitted, and the saccharose concentration was increased to 5%. Pyrex test tubes (diameter 20 mm) each containing 15 ml of medium were used. All media were autoclaved at 115°C for 20 min. To avoid dehydration during the 3-6 week culture period and to reduce the number of infections, the test tubes were covered firstly with cotton plugs, followed by aluminium foil and then finally Vitafilm (Good Year). Rhizome cultures were placed at 21°C under a schedule of 8 h photoperiod provided by fluorescent tubes (Philips TL 54/38 W, 6-8 Wm^{-2}) and 16 h darkness. Rooting experiments were carried out at 21°C and a light/dark schedule of 16 h fluorescent light/8 h darkness. In one experiment cultures were placed at different irradiances, whereas in another experiment the temperature was varied.

All experiments had one variable factor (nutritional, hormonal, physical, etc.). During rhizome culture each treatment consisted of 24 rhizome explants. The rhizome multiplication experiments were usually carried out with explants having one shoot (decapitated at isolation to a length of 0.5 cm) and one axillary bud. All explants used in the rooting experiments (with 12 rhizome explants per treatment) were derived from repeatedly subcultured rhizome explants; each explant consisted of a rhizome segment with 2-3 buds/shoots from which the

upright growing shoots were cut back to a length of 0.5 cm. Before transplanting rooted explants to soil, they were washed with tap water to remove agar, etc. In the greenhouse young plantlets were shaded and grown under plastic cover to maintain a relative high humidity. During the first weeks in soil, plants were sprayed periodically with Previcur-N (Schering) 0.2% to prevent contamination. All experiments reported were repeated at least once between the years 1985-1987. Data presented are the means of at least 2 experiments.

After 4 weeks, rhizome multiplication was evaluated by measuring the length of the upright growing shoots, and by counting the number of shoots and separable (for subculturing) rhizome explants. The parameter RMR (rhizome multiplication rate) represents the number of separable rhizome explants (with one bud or shoot) produced in 4 weeks, that are suitable for further subculturing. Rooting in vitro was evaluated after 6-7 weeks by determining the percentage rooting, the mean number of roots per explant and the mean root length.

3. Results

3.1. Rhizome multiplication

3.1.1. General

At the isolation of rhizome tips quite a number of cultures became infected due to internally present micro-organisms. This is a very difficult problem to overcome.

Numerous preliminary experiments were carried out to determine the optimal medium and other conditions (light, temperature) as described in the material and methods. The choice of our standard conditions was mainly based on results obtained with the cv 'Toledo', although it cannot be assumed that other cultivars have exactly the same requirements (e.g. cytokinin; see results).

3.1.2. Temperature

Table 1 - The influence of temperature on rhizome multiplication. MNUGS=mean number of upright growing (elongated) shoots per explant after 4 weeks. MLUGS=mean length (cm) of the upright growing shoots per explant after 4 weeks. RMR=rhizome multiplication rate after 4 weeks. RMR followed by the same letter means not significantly different at 5% level; RMR followed by a different letter means significantly different at 5% level. Mean separation by Student's T test.

Temperature (°C)	MNUGS	MLUGS	RMR
15	2.6	1.0	2.6a
18	2.3	1.1	2.6a
21	2.3	1.3	2.7a
24	2.0	1.2	2.2b

Table 1 shows that RMR (rhizome multiplication rate) was not affected by temperature in the range 15-21°C; only significant inhibition of the RMR occurred at 24°C in comparison with 15, 18, and 21°C. At 18°C less exudation of black/brownish substances occurred in comparison to 21 and 24°C.

3.1.3. Light

The irradiance range tested (1.5-9.7 W/m^2) had no significant effect on RMR (results not shown); only shoot length decreased by increasing the irradiance from 1.5-6.0 W/m^2. Despite the fact that A l s t r o e m e r i a is sensitive for growth and flowering to daylength, it was demonstrated (results not shown) that RMR was not significantly influenced by daylength (a comparison was made between 8 and 16 h at an irradiance of 6-8 W/m^2). Even when 2 multiplication cycles of 8 h (16 h) light were given, followed by one cycle of 16 h (8 h) darkness no differences in RMR were found.

When at the beginning or at the end of the multiplication cycle a period of 1-2 weeks darkness (also occurring in soil) was given, followed by light, the RMR was unaffected (results not shown).

3.1.4. Plant factors

A large variability was observed in RMR within each treatment. To see whether the original position of the explant was a source of this variation, apical and lateral rhizome explants (in our experiments normally mixed at random) were compared. Table 2 shows that apical tips had a signicantly higher RMR than lateral tips.

Table 2 - The influence of the position of the explant on rhizome multiplication.

Position	MNUGS	MLUGS	RMR
Apical	2.9	1.0	3.3a
Lateral	1.7	1.6	2.5b

Table 3 shows that the number of buds/shoots per explant strongly influenced RMR. By increasing the number of buds/shoots from 1-3, shoot number and RMR per bud/shoot decreased, whereas the length of the shoots increased. The most efficient way to multiply A l s t r o e m e r i a is is without doubt the single bud/shoot explant method.

Table 3 - The influence of the number of buds/shoots per explant on rhizome multiplication. MNUGS, MLUGS and RMR are always calculated per bud/shoot.

Number of buds/shoots per explant	MNUGS	MLUGS	RMR
1	2.2	0.8	2.4a
2	1.7	1.5	2.0b
3	1.2	1.6	1.4c

Table 4 shows that under the conditions optimal for the cv 'Toledo' the response of the other cultivars (hybrids) is quite different.

Table 4 - The influence of cultivar on rhizome multiplication. Each cultivar is grown on its own optimal BA concentration.

Cultivar	BA level (mg/l)	MNUGS	MLUGS	RMR
W.Fleming	4	2.4	1.2	2.9
Mendoza	2	2.4	2.3	2.8
Toledo	2	2.1	1.4	2.7
Paloma	4	1.9	1.6	2.3
Flamengo	3	1.7	1.3	1.9
Carmen	4	1.4	1.2	1.5
Tiara	4	1.2	1.0	1.2
Libelle	4	1.8	1.4	1.1

3.1.5. Length of the multiplication cycle

Table 5 shows that the multiplication rate obtained after a total of 15 weeks culture and after various lengths of the multiplication cycle was optimal when a cycle of 3 weeks was given 5 times. By increasing the length of the multiplication cycle RMR strongly declined.

Table 5 - The influence of the length of the multiplication cycle and the number of cycles on rhizome multiplication.

Cycles Number	weeks per cycle	MNUGS	MLUGS	Total RMR
5	3.00	1.3	1.1	11.7a
4	3.75	1.8	1.2	10.2b
3	5.00	2.3	1.5	9.1c
2	7.5	2.6	1.7	7.2d

3.1.6. The basic culture medium

Table 6 demonstrates that the concentration of macro-salts only significantly decreased the RMR at low levels (0.5 and 0.75) or at the highest level (2.0). When the ammonium nitrate concentration (0, 825, or 1650 mg/l) or the potassium nitrate concentration (0, 950, or 1900 mg/l) was varied no significant influence of the concentration was observed (results not shown).

Variation in the NaFeEDTA concentration (6.25, 12.5, 25, 37.5, 50 mg/l) did not influence the RMR significantly; higher levels (75 and 100 mg/l) of NaFeEDTA significantly decreased the RMR (results not shown).

The pH of the medium (a comparison was made between pH 5.0, 6.0, and 7.0) had virtually no effect on RMR (results not shown). A similar

result was obtained when the influence of the agar concentration (0.5, 0.6, and 0.7) was tested (results not shown).

Table 6 - The influence of the MS-macro-salt strength on rhizome multiplication.

MS-salts (strength)	MNUGS	MLUGS	RMR
0.50	2.5	1.1	2.0a
0.75	2.9	1.1	2.3a
1.00	3.0	1.4	2.6b
1.25	3.1	1.2	2.9b
1.50	3.0	1.2	2.6b
2.00	2.5	1.2	2.1a

3.1.7. Carbohydrates

Preliminary experiments demonstrated that a higher RMR was obtained with saccharose than with glucose (results not shown). Table 7 shows that the saccharose concentration markedly influenced the RMR, 3-4% being optimal.

Table 7 - The influence of the saccharose concentration on the rhizome multiplication.

Saccharose (%)	MNUGS	MLUGS	RMR
1.0	1.9	0.7	2.0a
2.0	3.0	0.9	2.3a
2.5	2.7	1.1	2.3a
3.0	3.0	1.4	2.7b
3.5	2.8	1.7	2.9b
4.0	2.8	1.5	2.7b
5.0	3.0	1.3	2.4a

Table 8 shows the results of a comparison between solid and liquid medium; in this experiment much lower BA levels were chosen in the liquid media than used in solid media because relative high BA levels were toxic in the liquid media. MNUGS, MLUGS, and RMR appeared to be higher in liquid media. However, repeated rhizome multiplication on liquid media resulted in vitrification which is very detrimental.

Table 8 - The influence of BA at 2 mg/l in a solid medium compared with various BA levels in liquid media on rhizome multiplication.

Medium	BA conc. (mg/l)	MNUGS	MLUGS	RMR
Solid	2.00	2.3	1.2	2.3a
Liquid	0.10	2.6	5.6	2.7ab
,,	0.25	3.2	4.7	3.1bc
,,	0.50	3.4	4.2	3.3c
,,	1.00	3.7	3.2	3.4c

Addition of complex mixtures of substances such as peptone (0.5 or 1.0 g/l), tryptone (0.5 or 1.0 g/l), casein hydrolysate (200 or 500 mg/l), banana homogenate (30 or 80 g/l), or coconut milk (50 or 100 ml/l) had no significant effect on RMR. Peptone at 1.0 g/l significantly decreased RMR (results not shown); the same holds true for banana homogenate at 80 g/l.

3.1.8. Regulators

Abbreviations used:

BA=6-benzylaminopurine
IAA=indole-3-acetic acid
IBA=indole-3-butyric acid
2iP=6-(γ γ)-dimethylallylamino)purine
NAA=α-napthaleneacetic acid
PBA=6-(benzylamino)-9-(2tetrahydropyranyl)-9H-purine
zeatin=6-(4-hydroxy-3-methyl-2-butenylamino)purine

NAA (concentrations tested: 0, 0.01, 0.1, and 1.0 mg/l) and IBA (concentrations tested: 0, 0.01, 0.1, 0.5, and 1.0 mg/l) had no promotory effect on RMR. NAA (at 1.0 mg/l had a significant inhibitory effect on RMR (results not shown).

Table 9 shows that the addition of BA strongly influenced the MNUGS, the MLUGS, and the RMR. The MNUGS strongly increased with the addition of BA with an apparent optimum (not significant) at 2.0 mg/l. The MLUGS strongly decreased with increasing BA concentration, whereas the RMR increased up to a BA concentration of 2.0 mg/l and then decreased. At a concentration of 8 mg/l BA, shoots and rhizomes were strongly deformed.

Table 10 shows the influence of various 2iP and kinetin concentrations in comparison with BA at 2.0 mg/l. Table 11 shows the effect of various concentrations of zeatin riboside. Tables 10 and 11 show that BA is a much more effective cytokinin than the other three tested. Preliminary experiments (results not shown) with PBA showed that at concentrations of 2 to 5 mg/l it was equally as effective as BA.

Table 9 - The influence of the BA concentration on rhizome multiplication.

Cytokinin	Conc. (mg/l)	MNUGS	MLUGS	RMR
BA	0	1.1	3.7	1.0a
	0.5	2.7	2.0	2.3b
	1.0	3.3	1.5	2.4b
	2.0	3.0	1.3	2.7b
	4.0	3.0	1.1	2.5b
	8.0	2.3	0.8	2.2b

Table 10 - The influence of the BA, 2iP and kinetin concentration on rhizome multiplication.

Cytokinin	Conc. (mg/l)	MNUGS	MLUGS	RMR
No	0	1.1	3.5	1.0a
Kinetin	0.5	1.2	4.4	1.2b
,,	2.0	2.0	2.9	1.9c
,,	8.0	2.8	1.8	2.1c
2iP	0.5	1.2	4.2	1.2b
,,	2.0	1.2	3.3	1.3b
,,	8.0	1.3	3.2	1.4b
BA	2.0	2.8	1.2	2.7d

Table 11 - The influence of zeatin riboside and BA on rhizome multiplication.

Cytokinin	Conc. (mg/l)	MNUGS	MLUGS	RMR
Zeatin rib.	0.5	2.2	2.9	1.6a
,,	1.0	2.3	3.0	1.9a
,,	2.0	2.5	2.5	1.9a
,,	4.0	2.3	2.9	2.1a
BA	2.0	2.5	1.7	3.0b

3.2. Rooting

The results of our rooting experiments with the cv 'Toledo' can be summarized as follows:

- Rooting was promoted by a daylength of 16 h in comparison to 8 h.
- An irradiance of 7 W/m^2 was optimal; rooting strongly decreasing at lower (1.2-2.2 W/m^2) or higher irradiances (15.4 W/m^2).
- An initial dark treatment (1, 2, or 3 weeks) had a negative effect on rooting.
- Optimal rooting (% of rooting, mean number of root primordia per explant) occurred at 21°C in comparison to 25°C and 27°C. At 25°C rooting decreased and the quality (colour) of the rooted plantlets was much lower (paler) than at 21°C.
- A MS macro-salts strength of 1.0 was optimal in comparison to 0.25 or 0.50.
- A pH of 6.0 was optimal in comparison to 5.0 and 7.0.
- Glucose was less effective than saccharose. Optimal rooting occurred at 5% saccharose.
- A comparison of IBA and NAA, tested in the concentration range of 0, 0.1, 0.5, 1.0, 2.0, and 5.0 mg/l showed that NAA was generally more effective than IBA; the optimal concentration being 0.5 mg/l.

- Cytokinin should be ommitted after rhizome multiplication, to induce rooting.

From preliminary experiments with 3 other cultivars, the following was concluded:
- NAA was more effective on rooting in the cultivars Mendoza (optimal concentration 0.25-0.50 mg/l) and Paloma (optimal concentration 0.75 mg/l) than IBA.
- For the cultivar Walter Fleming IBA (optimal concentration 0.5 mg/l) was slightly more effective than NAA.

In vitro rooted plantlets, from which the upright growing shoots were removed, were successfully transferred to soil (greenhouse, under an extra plastic cover to reduce desiccation). All in vitro produced plants flowered normally.

4. Discussion

From our experiments it is clear that at least 8 factors determine the rhizome multiplication of A l s t r o e m e r i a: temperature, cultivar, number of buds/shoots per explant, duration of the multiplication cycle, concentration of macro-salts, concentration of saccharose, solid/liquid medium, an effective cytokinin. In previous publications (Gabryszewska and Hempel, 1985; Lin and Manette, 1987) on A l s t r o e m e r i a rhizome multiplication, these requirements were not completely fulfilled. Two additional factors not studied in our research are: pre-treatment of the mother plants at low temperature (Lin and Manette, 1987), and the possible role of auxin on cultivars which are difficult to clone.

Rooting was also influenced by a number of factors, some of which have not previously been reported. It is possible that the rooting requirements for other cultivars are different to those optimal for the cv 'Toledo'.

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