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Regeneration of leaf explants of Anthurium andraeanum Lind. in vitro*

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Summary

The effect of various factors on callus formation on excised leaf explants of *Anthurium andraeanum* was studied; special attention was paid to the regeneration of sprouts in this callus. A strong positive correlation was found between callus formation and the regeneration of sprouts. Adventitious sprout formation was optimal under the following conditions: addition of 0.25-1.00 ml/litre Tween 20 during leaf sterilization, adenine 0.1 mg/litre, zeatin 1 mg/litre, 2,4-D 0.08 mg/litre, culture during 16 weeks in darkness followed by 4 weeks of light, 206 mg/litre NH_4NO_3 in the medium. It was shown that the promoting effect of low levels of NH_4NO_3 on sprout regeneration in callus is caused by the NH_4^+ ion and not by the NO_3^- ion.

Introduction

In a previous paper on vegetative propagation of *Anthurium andraeanum* in vitro, particular attention was paid to the formation of callus on leaf explants, the subculture of callus on solid and liquid media, the regeneration of adventitious sprouts and the regeneration of roots on excised sprouts (Pierik, 1976). It was shown that for the induction of adventitious sprouts in callus tissues a low NH_4NO_3 concentration in the culture medium is a very essential factor.

The present paper deals with the influence of several factors on the formation of callus and regeneration of adventitious sprouts in the callus on leaf explants in a system without callus subculturing.

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Materials and methods

The experiments in vitro were done with leaf explants of a two-year-old flowering *Anthurium andraeanum* Lind. clone (K8) grown in the greenhouse. Selected leaves were relatively young, soft and just expanding. Leaves were sterilized for a few seconds in alcohol 70 %, 10 minutes in 1 % NaOCl (10 % commercial bleaching liquor) with 0.25 ml/litre of Tween 20 and then rinsed 3 times in sterile tap water for 3, 10 and 20 minutes. Leaf explants of 1.5 cm^2 were cut excluding the mid vein and were randomly distributed over the treatments. Square leaf explants with basal ends down were placed in solid media to a depth of about half their diameter. The number of explants per treatment was 24. The experiments were carried out from April to September 1978. Test tubes were closed with cotton plugs (without burning) and aluminium foil. Unless otherwise stated test tubes were placed at 25 °C in continuous darkness.

The composition of the basic culture medium (= control in the tables) was as follows: NH_4NO_3 206, KNO_3 950, $CaCl_2 \cdot 2H_2O$ 440, $MgSO_4 \cdot 7H_2O$ 370, KH_2PO_4 85 (all in mg/l), Murashige & Skoog's (1962) microelements (except the Fe source), NaFeEDTA 25, nicotinic acid 0.5, pyridoxin 0.5, thiamin 0.1, glycin 2.0, meso-inositol 100, 6-benzylamino-purine (BA) 1.0, 2,4-D 0.08 (all in mg/l), glucose 3 % and Difco Bacto agar 0.7 %. The choice of this medium was based on the results described earlier (Pierik, 1976). The pH of the media was adjusted to 6.0 before autoclaving.

At the end of each experiment, 20 weeks after isolation, the following observations were made: the percentages of callus formation and sprout regeneration, the number of sprouts (longer than 1 mm) per explant, the fresh weight of callus and sprouts per explant, root regeneration. Because root initiation and development were very poor under all conditions, no data on root regeneration are presented in the tables. Since the percentage of callus regeneration was almost 100 % in all treatments, these percentages are omitted in the tables. The average number of sprouts per explant and the mean fresh weight of callus and sprouts per explant were calculated, using all non-infected explants. The average infection percentage was 12.

Results

In all experiments the formation of callus at the margins of the leaf explants preceded the regeneration of sprouts. Sprout regeneration without any callus formation was never observed and callus formation should therefore be considered as a prerequisite for sprouting, which always occurred in the callus.

In some preliminary experiments the effects of the age of the plants, the age of the leaves and the influence of the burning of the cotton plugs were analysed. The results of these experiments can be summarized as follows. A comparison of young expanding leaves from plants 2 and 5 years old of the same clone demonstrated that the capacity to form callus and sprouts in this callus decreased by using leaves of 5-year-old plants. When leaves in various stages of development

Table 1. The effect of adenine, 4 cytokinins and 2,4-D in various concentrations on callus formation and sprout regeneration after 20 weeks on leaf explants of *Anthurium andraeanum*. Control = *.

Experiment	Compound	Concentration (mg/litre)	Sprout regeneration (%)	Mean number of sprouts per explant	Fresh weight of callus and sprouts per explant (g)
1	Adenine	0*	100	11.3	0.95
		0.1	100	17.0	1.10
		1	90	15.0	1.07
		10	86	12.5	1.21
		50	70	6.3	1.17
2	BA	0.05	27	0.4	0.16
		0.5	95	12.1	0.80
		1.0*	86	10.8	1.01
		10.0	0	0.0	0.07
2	Kinetin	0.05	0	0.0	0.10
		0.5	71	3.5	0.29
		1.0	83	8.5	0.56
		10.0	0	0.0	0.09
2	2-iP	0.05	0	0.0	0.07
		0.5	9	0.1	0.18
		1.0	77	2.8	0.27
		10.0	82	4.8	0.68
2	zeatin	0.05	0	0.0	0.12
		0.5	87	11.7	0.48
		1.0	96	25.5	1.07
		10.0	79	8.3	1.41
3	2,4-D	0	57	5.4	0.61
		0.08*	86	7.0	0.61
		0.1	88	5.3	0.55
		0.5	10	0.1	0.30
		1.0	5	0.1	0.15

of two year old plants were compared, it soon became clear that just unfolded leaves, which were very soft, had the highest regeneration capacity; a similar reaction was observed when various leaves of 5-year-old plants were compared. A severe effect of cotton burning became evident in our first series of experiments; it was shown that cotton plug burning was very detrimental for the survival and the regeneration of the leaf explants. Burning induced first a yellowing of the leaf explants above the medium, followed by browning and necrosis which was unfavourable for regeneration. For that reason cotton burning was immediately stopped and it was supposed that the burning of cotton plugs produced toxic substances.

In a number of experiments without cotton burning and with young leaves of

Table 2. The effect of the Tween 20 concentration and various dark-light regimes on callus formation and sprout regeneration after 20 weeks on leaf explants of *Anthurium andraeanum*. Control = *.

Experiment	Tween concentration (ml/litre)	Weeks dark + weeks light	% Sprout regeneration	Mean number of sprouts per explant	Fresh weight of callus and sprouts per explant (g)
4	0	20 + 0*	88	4.1	0.59
	0.25*		86	7.0	0.61
	0.50		91	8.2	0.65
	1.00		87	7.1	0.83
5		20 + 0*	89	16.4	1.03
		16 + 4	100	19.2	1.20
		12 + 8	95	18.0	1.14
		8 + 12	100	17.4	1.25
		4 + 16	85	14.3	0.91
		0 + 20	35	1.4	0.39

a 2-year-old clone the formation of callus and regeneration of sprouts was further analysed. The results of these experiments are shown in Tables 1-3. In each experiment the standard treatment (= control), as described in the methods, is compared with variations of a single factor (such as Tween 20 conc. during

Table 3. The effect of the NH_4NO_3, $(NH_4)_2SO_4$ and $NaNO_3$ concentration on callus formation and sprout regeneration after 20 weeks on leaf explants of Anthurium andraeanum. Control = *.

Experiment	Compound	Concentration (mg/litre)	% Sprout regeneration	Mean number of sprouts per explant	Fresh weight of callus and sprouts per explant (g)
6	NH_4NO_3	0	65	4.1	0.46
		206*	95	9.4	0.86
		412	38	1.7	0.51
		618	41	1.8	0.54
		825	26	0.7	0.39
7	NH_4NO_3	206*	84	7.3	0.86
	$(NH_4)_2SO_4$	0	77	4.1	0.55
		85	86	7.6	0.78
		170	84	6.1	0.81
		341	48	1.9	0.49
		681	20	0.2	0.23
7	$NaNO_3$	0	81	4.0	0.44
		110	65	3.0	0.41
		219	64	2.8	0.38
		439	62	1.7	0.43
		877	47	1.4	0.33

sterilization, adenine conc., cytokinin conc., 2,4-D conc., dark/light regime, conc. of NH_4NO_3, $(NH_4)_2SO_4$ and $NaNO_3$).

A promoting effect of adenine on the mean number of sprouts per explant, as demonstrated by Welles (unpublished), was reexamined. Table 1 (exp. 1) shows that at low adenine concentrations (0.1-1.0 mg/litre) the mean number of sprouts per explant is strongly promoted as compared with the control with the best reaction at 0.1 mg/litre, whereas a decrease of sprout regeneration is observed when the adenine conc. is increased from 0.1 to 50 mg/litre. Since the lowest concentration used was 0.1 mg/litre, it cannot be concluded that at this concentration the optimum is already reached. A comparison of 4 cytokinins in various concentrations (Exp. 2) shows that optimal callus and sprout formation occurs when zeatin is added at 1 mg/litre, followed by BA at 1 mg/litre, kinetin at 1 mg/litre and 2-iP (N6-isopentenyl-adenine) at 10 mg/litre. Since the highest 2-iP concentration was 10 mg/litre, it cannot be concluded that at 10 mg/litre 2-iP the optimum is already reached. A promotion of sprout formation is also observed when 2,4-D is added to the culture medium (Exp. 3) with an optimum at 0.08 mg/litre.

In earlier experiments (Scheurink & Welles, unpublished) a promoting effect on sprout regeneration of Tween 20 during leaf sterilization was observed. Table 2 (Exp. 4) shows that the number of adventitious sprouts is increased when Tween 20 is added at 0.25-1.00 ml/litre. Although the regeneration and development of sprout primordia in *Anthurium* callus is generally promoted by darkness, the effect of a light period after an initial dark period was examined. Exp. 5 shows that continuous light during 20 weeks (0 + 20) is strongly inhibiting for regeneration, whereas continuous darkness during 20 weeks (20 + 0) strongly induces sprout regeneration. But it is also clear that combinations of 16 weeks darkness + 4 weeks light, 12 + 8 or 8 + 12 are the most optimal dark-light regimes for regeneration. An explanation for the relative high number of sprouts per explants in the control of Exp. 5, as compared with the other controls, cannot be given.

In several earlier experiments (Pierik, 1976; Scheurink & Welles, unpublished) a promoting effect of low NH_4NO_3 concentrations on sprout regeneration in subcultured callus and leaf explants was demonstrated. Table 6 (Exp. 6) shows that also in excised leaf explants a promoting effect of low NH_4NO_3 levels can be demonstrated, whereas higher levels were inhibitory. The question arose whether the promoting effect of low NH_4NO_3 levels is caused by the NH_4^+ and/or the NO_3^- ion. For that reason an experiment was designed in which NH_4NO_3 was replaced by either $(NH_4)_2SO_4$ (only the NH_4^+ ion from NH_4NO_3 present) or $NaNO_3$ (only the NO_3^- ion from NH_4NO_3 present). A concentration of 170 mg/litre $(NH_1)_2SO_4$ in Table 3 (Exp. 7) corresponds with an equivalent conc. of NH_4 in 206 mg/litre NH_4NO_3, whereas a concentration of 219 mg/litre $NaNO_3$ corresponds with a equivalent concentration of NO_3 in 206 mg/litre NH_4NO_3. Exp. 7 shows that the sprouting optimum is reached at a $(NH_4)_2SO_4$ concentration of 85 mg/litre, whereas sprout formation is decreased by a further increase of the $(NH_4)_2SO_4$ concentration. When the $NaNO_3$ range is considered, it can be concluded that over the whole concentration range sprouting is decreased by

increasing the $NaNO_3$ level. In conclusion, it can be stated that the promoting effect of low nitrogen levels on sprouting can only be caused by the NH_4^+ion and not by the NO_3^-ion.

Conclusion and discussion

From the foregoing experiments it is quite clear that the regeneration of leaf explants of *Anthurium andraeanum* clone K8 is limited both qualitatively and quantitatively by a complex of factors: plant, environmental, nutritional and hormonal. When certain requirements are not fulfilled, sprout formation is almost inhibited or strongly reduced. The principle of limiting factors is therefore certainly applicable in the present case. To achieve optimum sprouting in callus, it is necessary to know just how to balance many factors described and to make the results valuable for as many cultivars as possible. It was already demonstrated that several other cultivars of Anthurium andraeanum at low NH_4NO_3 levels are also capable to regenerate sprouts from leaf explants, but it appears that the sprouting capacity is strongly cultivar dependent. It is reasonable to suppose that when all factors examined are optimal that sprout regeneration in more cultivars can effectively be induced.

The results obtained with adenine, cytokinins and 2,4-D are generally in accordance with the literature on sprout regeneration (cf. Pierik, 1975). The promoting efect of darkness on callus formation is as expected but the promoting effect of darkness on sprout regeneration is rather unusual. The role of NH_4NO_3 and $(NH_4)_2SO_4$ and particularly the role of the NH_4^+ion (promotion of sprouting at low levels and inhibition of sprouting at high levels) forms the most puzzling fact brought to light in this paper. An explanation for the NH_4^+ effect cannot be given yet.

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