Plant Cell Reports (1995) 14:520–524

Plant Cell Reports

In vitro propagation of taro, with spermine, arginine and ornithine. II. Plantlet regeneration via callus

Shakuntala Sabapathy and Helen Nair

Department of Botany, University of Malaysia, 59100 Kuala Lumpur, Malaysia

Received 27 September 1993/Revised version received 24 October 1994 – Communicated by J. M. Widholm

Abstract

Regeneration of plantlets from shoot apex-derived callus and "calloid" cultures of a local taro [*Colocasia esculenta* var. *antiquorum* cv. Keladi Birah] cultivar, was expedited by treatment with high levels of spermine. The total time taken, from culture of primary shoot apices on modified Linsmaier and Skoog medium supplemented with trichlorophenoxyacetic acid and kinetin, to complete plantlet regeneration, was reduced by 2–16 weeks, when the callus and "calloid" cultures were treated with 0.01, 0.1 and 1 mM spermine. Furthermore, the number of plantlets produced per gram callus increased from 25 to 55. On media supplemented with arginine and ornithine, no callus was initiated from explants and no plantlets differentiated from pre-established callus.

Key words: Arginine-callus–*Colocasia esculenta* var. *antiquorum*–plantlet regeneration–ornithine–spermine–taro

Introduction

Taro [*Colocasia esculenta* (L.) Schott], besides being a staple food crop in certain regions of the world, has other useful applications: namely as fodder for animals, raw material for the industrial production of alcohol and plastics as well as for traditional medicines and religious purposes. Micropropagation of taro from primary shoot apices and axillary buds, as well as via callus, has been documented for cultivars belonging to both *C. esculenta* var. *antiquorum* [Abo El-Nil and Zettler, 1976; Nyman *et al.*, 1983; Nyman and Arditti, 1984; Malamug *et al.*, 1992 a,b; Sabapathy and Nair, 1992] and *C. esculenta* var. *esculenta* [Irawati and Webb, 1984; Yam *et al.*, 1991]. However, the process of plantlet regeneration via the callus pathway remains tediously slow. In this paper, we expand on an earlier study [Sabapathy and Nair, 1992] and report the effects of polyamine spermine and polyamine precursors, arginine and ornithine, on in vitro morphogenesis of a local taro cultivar, *C. esculenta* var. *antiquorum* cv. Keladi Birah in callus-raised cultures.

Correspondence to: S. Sabapathy

Materials and methods

Plant material. Primary shoot apices were obtained aseptically from a local taro cultivar [*C. esculenta* var. *antiquorum* cv. Keladi Birah] as reported earlier [Sabapathy and Nair, 1992].

Culture media. Explants were cultured on Linsmaier and Skoog medium (LS) containing 0-3 mg l^{-1} trichlorophenoxyacetic acid [2,4,5-T] and 0–0.2 mg l^{-1} kinetin [LSC3] for callus initiation. The initiated callus was transferred to LS supplemented with 4-6 mg l^{-1} naphthaleneacetic acid [NAA] and 0–0.2 mg l^{-1} kinetin [LSC4], for proliferation. After substantial proliferation (until maximum proliferation had taken place and there was no further development after 11 weeks in this medium) the callus was placed on differentiation media comprising of LS plus 0–2 mg l^{-1} kinetin [LSC5], for plantlet regeneration.

Treatments with spermine, arginine and ornithine. Each of the three culture media, LSC3, LSC4 or LSC5 was enriched with various levels of spermine, arginine or ornithine [Table 1] to examine morphogenetic responses. These filter-sterilized additives were utilized only in the initial treatments. They were not added thereafter to the subculture media. All the other cultural procedures were as reported earlier [Sabapathy and Nair, 1992].

Results and discussion

Callus initiation, proliferation and plantlet regeneration On LSC3, LSC4 and LSC5

Callus was initiated on LSC3 medium containing 2 mg l^{-1} 2,4,5-T and 0.2 mg l^{-1} kinetin and proliferated substantially on transfer to LSC4, containing 5.5 mg l^{-1} NAA and 0.2 mg l^{-1} kinetin. The proliferated callus consisting of yellow-white, nodular tissues surrounded by friable, pale yellow, translucent masses of cells [Fig. 1A, B], when transferred to LSC5 medium provided with 2 mg l^{-1} kinetin, underwent transition to patches of opaque, dark green, meristematic "calloid" tissue. The tissue was similar to that described earlier by Nyman *et al.* [1983] as consisting of "not a homogenous callus" but of "young shoots, as well as fasciated or determinate shoot-like and leaf-like structures" [Fig. 1C]. Normal shoots differentiated from the "calloid" tissue by week 28 in the same medium [Fig. 1D] and fully developed plantlets were obtained after 45 weeks [Fig. 1E; Table 1]. Thereafter, these were

Table 1: Regimes for plantlet regeneration from spermine-treated cultures of taro [*C. esculenta* var *antiquorum* cv. Keladi birah]

Media	Tissue in Culture	Developmental Response[a]	Total Time in Culture[b] (weeks)	Plantlets per g Callus[c]
Controls[d]				
LSC3	Shoot apex	Callus initiation	7	
LSC4	Initiated callus	Callus proliferation	18	
LSC5	Proliferated callus	Calloid formation	22	
		∇		
		Shoot production	28	
		∇		
		Full development of plantlets	45	24 ± 3
Spermine treatments[e]				
LS3 + 0.01 mM Spn	Shoot apex	Callus initiated but no further growth on transfer to LSC3 and LSC4	7	
LS3 + 0.1 mM Spn	Shoot apex	Callus initiated but no further growth on transfer to LSC3 and LSC4	7	
LS3 + 1.0 mM Spn	Shoot apex	Callus initiation	10	
		∇		
		LSC3 (No development)	18	
		∇		
		LSC4		
		↓		
		Shoot production	25	
		↓		
		Full grown plantlets	35	28 ± 5
LS4 + 0.01 mM Spn	Initiated callus	Callus proliferated but no further development on transfer to LSC4	18	
LS4 + 0.1 mM Spn	Initiated callus	Callus proliferated but no further development on transfer to LSC4	18	
LS4 + 1.0 mM Spn	Initiated callus	Callus proliferation	18	
		∇		
		LSC4		
		↓		
		Shoot production	22	
		↓		
		Fully developed plantlets	29	31 ± 6
LS5 + 0.01 mM Spn	Proliferated callus	No development in this medium and after transfer to LSC5	22	
LS5 + 0.1 mM Spn	Proliferated callus	No development observed in this medium	24	
		∇		
		LSC5		
		↓		
		Shoot production	28	
		↓		
		Full grown plantlets	33	30 ± 4
LS5 + 1.0 mM Spn	Proliferated callus	No development observed in this medium	24	
		∇		
		LSC5		
		↓		
		Shoot production	26	
		↓		
		Full grown plantlets	30	42 ± 6
LS5 + 0.01 mM Spn	Calloid mass	No development seen in this medium	25	
		∇		
		LSC5		
		↓		
		Shoot production	30	
		↓		
		Full grown plantlets	43	27 ± 3
LS5 + 0.1 mM Spn	Calloid mass	No development seen in this medium	25	
		∇		
		LSC5		
		↓		
		Shoot production	28	
		↓		
		Full grown plantlets	37	40 ± 5
LS5 + 1.0 mM Spn	Calloid mass	Shoots produced in this medium	25	
		∇		
		LSC5		
		↓		
		Full developed plantlets	34	55 ± 8

[a]Subculturing was carried out onto the appropriate control culture medium at 3-4 weeks interval
[b]Total number of weeks taken to attain this developmental stage, since beginning of culture
[c]Results are average of two trials, with 7 replicates/treatment
[d]LSC3 = Control Callus initiation medium (minus spermine)
LSC4 = Control Callus proliferation medium (minus spermine)
LSC5 = Control Plantlet regeneration medium (minus spermine)
[e]Explants used for spermine supplemented callus initiation, callus proliferation and plantlet regeneration media:
LS3 + Spn — Shoot apex LS4 +Spn — Initiated callus from LSC3. LS5 + Spn — Proliferated callus from LSC4 LS5 + Spn — Calloid mass from LSC5
Spn = Spermine ∇ Transfer to another medium

successfully established on soil [Fig. 1F]. Similar morphogenetic changes have also been reported for callus cultures of rice [Inoue and Maeda, 1980] and the antiquorum variety of taro [Nyman *et al.*, 1983] although not in the esculenta variety [Irawati and Webb, 1984; Yam *et al.*, 1991].

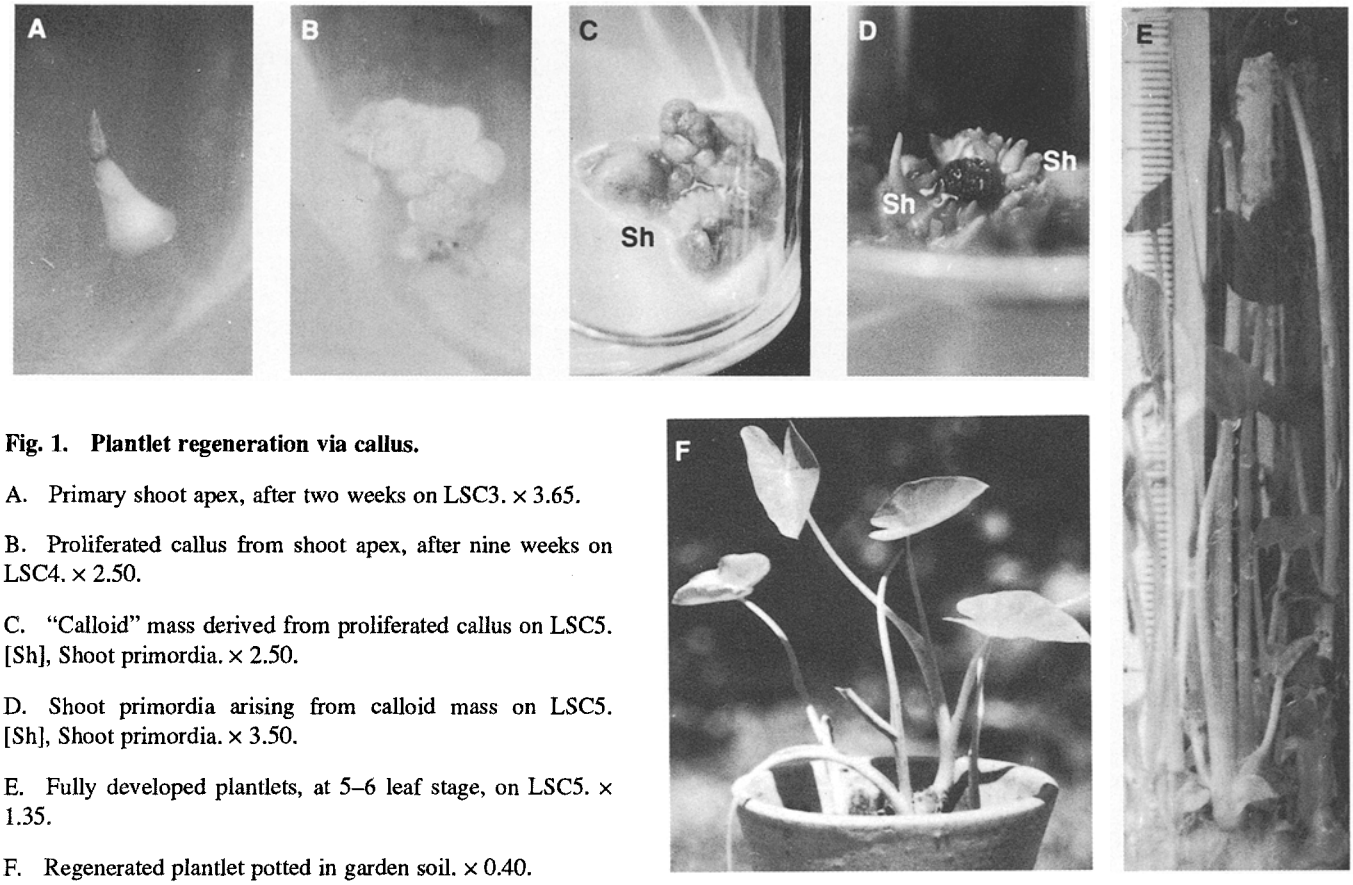

Fig. 1. Plantlet regeneration via callus.

A. Primary shoot apex, after two weeks on LSC3. × 3.65.

B. Proliferated callus from shoot apex, after nine weeks on LSC4. × 2.50.

C. "Calloid" mass derived from proliferated callus on LSC5. [Sh], Shoot primordia. × 2.50.

D. Shoot primordia arising from calloid mass on LSC5. [Sh], Shoot primordia. × 3.50.

E. Fully developed plantlets, at 5–6 leaf stage, on LSC5. × 1.35.

F. Regenerated plantlet potted in garden soil. × 0.40.

Effects of spermine on plantlet differentiation

On examining the effects of the polyamine spermine and precursors arginine and ornithine, only spermine was found efficacious. Arginine and ornithine were not effective either in initiating callus from explants or in differentiating plantlets from pre-established callus of taro. However, we have found that all the three additives individually enhanced direct plantlet regeneration from primary shoot apices, axillary buds and protocorm-like bodies of taro [Sabapathy and Nair, 1992].

On spermine-supplemented LSC3

Primary shoot apices, cultured on spermine-supplemented LSC3, initiated callus, although at a concentration of 1mM spermine, callus initiation was delayed by three weeks [Table 1]. However, on transfer to LSC4, shoots differentiated from cultures under this treatment by week 25 and plantlet regeneration was completed by week 35 [Fig. 2A; Table 1], without proceeding through a calloid stage. Hence a final transfer to regeneration medium, LSC5, was not necessary and the total culture time was reduced by 10 weeks. With 0.01 and 0.1 mM spermine treatments, callus was initiated, however, there was no further growth even after transfer to LSC4 medium (Table 1).

On spermine-supplemented LSC4

Callus masses initiated from primary shoot apices on LSC3, were placed on spermine-treated LS4 for proliferation. Shoots were produced, when well proliferating calli on 1 mM spermine-enriched medium were transferred to LSC4. Complete plantlets were obtained by week 29 [Table 1; Fig. 2B]. The calloid stage was also obviated in these cultures, thereby shortening the total culture time by 16 weeks. There was no further development in the proliferated calli, obtained under 0.01 and 0.1 mM spermine treatments, on transfer to LSC4 (Table 1).

On spermine-supplemented LSC5

When proliferated callus masses from LSC4, were cultured on 0.1 or 1 mM spermine containing LS5 and maintained therein for six weeks, then transferred to LSC5, shoots were produced. Fully grown plantlets were obtained between weeks 30 to 33 [Table 1; Fig. 2C]. If calloid masses derived from callus on LS5, were subcultured onto LS5 plus spermine, shoots were produced in the same medium at week 25 and after. Complete plantlets were formed by the 32nd to 37th week, in cultures under 0.1 and 1mM spermine treatments. While, in 0.01 mM

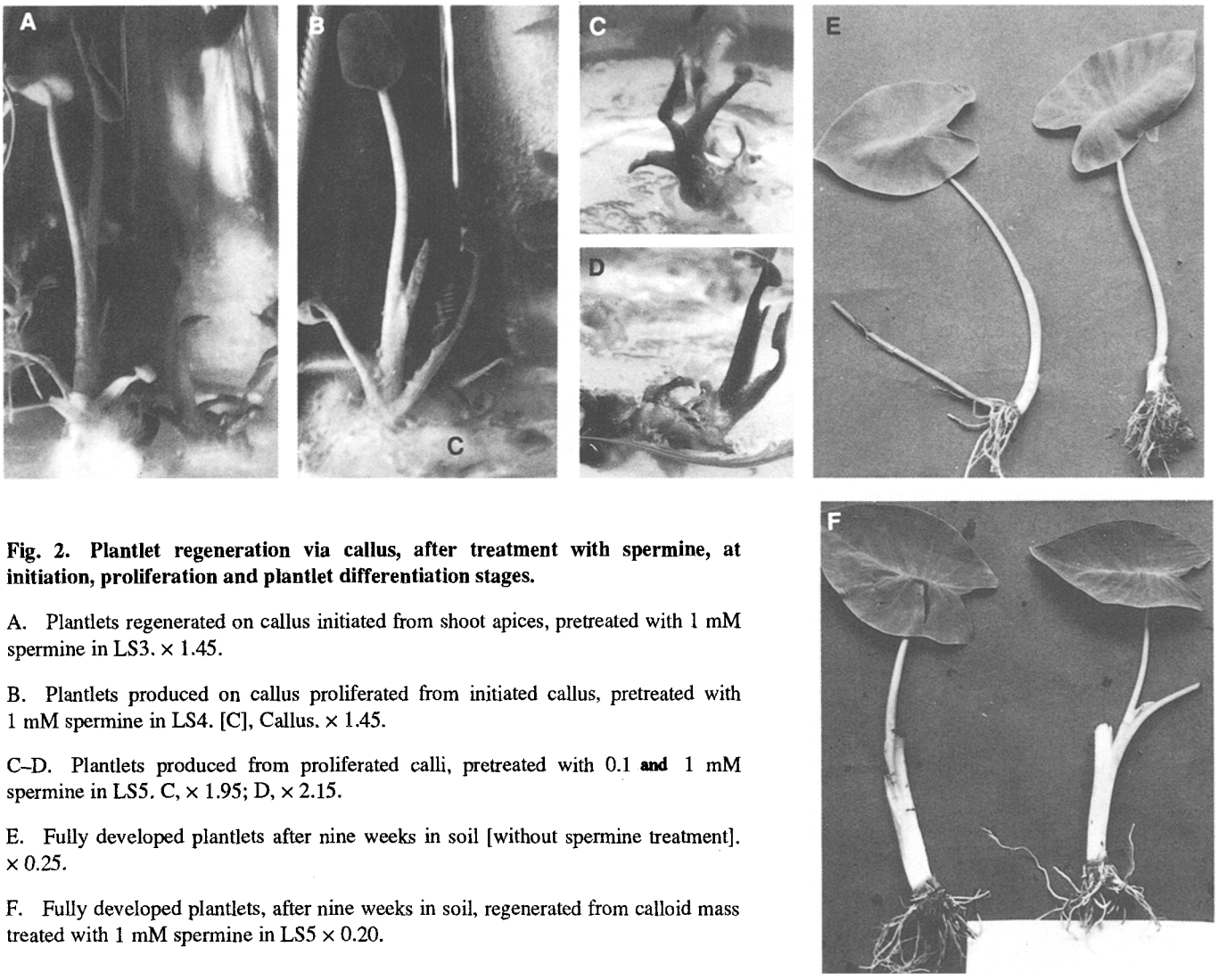

Fig. 2. Plantlet regeneration via callus, after treatment with spermine, at initiation, proliferation and plantlet differentiation stages.

A. Plantlets regenerated on callus initiated from shoot apices, pretreated with 1 mM spermine in LS3. × 1.45.

B. Plantlets produced on callus proliferated from initiated callus, pretreated with 1 mM spermine in LS4. [C], Callus. × 1.45.

C–D. Plantlets produced from proliferated calli, pretreated with 0.1 **and** 1 mM spermine in LS5. C, × 1.95; D, × 2.15.

E. Fully developed plantlets after nine weeks in soil [without spermine treatment]. × 0.25.

F. Fully developed plantlets, after nine weeks in soil, regenerated from calloid mass treated with 1 mM spermine in LS5 × 0.20.

spermine-supplemented medium, plantlets developed fully between 6 to 11 weeks later [Table 1; Fig. 2D].

The highest yields were obtained on LS5 medium supplemented with 1 mM spermine. Under this treatment, the calloid tissue yielded as many as 55 plantlets per g tissue. Furthermore, developmental time was 12 weeks shorter than in control cultures [Table 1]. The presence of spermine during the growth cycle was also conducive to the production of sturdy plantlets with large leaves and a good root system [Fig. 2 E, F].

Polyamines and their precursors are recognized as essential for proper growth and development in plants, animals and microorganisms [Tabor and Tabor, 1984; Galston and Smith, 1985; Evans and Malmberg, 1989]. Increased levels of spermine and other polyamines have also been reported to influence organogenesis and embryogenesis [Bradley *et al.*, 1984; Kaur-Sawhney *et al.*, 1988; Basu *et al.*, 1989; Ismail and D'Auzac, 1992; Sabapathy and Nair, 1992], although their mode of action remains unclear. Putrescine [a diamine], spermidine [a triamine] and spermine [a tetra-amine] occur in all higher plants. They are generally known to interact with the phosphate residues in nucleic acids and membranes, thus influencing the transcription-translation processes as well as membrane stabilization [Smith, 1985]. Apart from their direct effect on embryogenesis and plantlet formation, they are also known to enhance morphogenesis indirectly by acting as free-radical scavengers or anti-oxidants [Ismail *et al.*, 1993; Tiburcio *et al.*, 1993]. However, their role is not consistently the same. Hence often when putrescine promotes growth and differentiation, the higher homologs, spermidine and spermine, may not be effective or vice versa [Desai and Mehta, 1985]. Similarly, although arginine and ornithine were able to promote the initiation and development of secondary shoots from apical meristems of taro [Sabapathy and Nair, 1992], yet, in the present study, these precursors failed to induce callus or even differentiate plantlets from pre-formed callus tissue.

In this study, the use of spermine as a medium supplement for each developmental stage in taro culture, has resulted in a two-fold benefit: a substantially reduced regeneration time [by 2–16 weeks] and a concomitant increase in the number of plantlets produced. The best protocols appear to be those involving all the three media

– LSC3, LSC4 and LSC5. In these cases, the proliferated callus (obtained after callus initiation on LSC3 and proliferation on LSC4) and the calloid mass (obtained after callus initiation on LSC3, proliferation on LSC4 and after transition of this proliferated callus to calloid mass on LSC5), cultured separately on 1.0 mM spermine supplemented LS5 medium, produced full grown plantlets by weeks 30 and 34 and gave a maximum of 42 and 55 plantlets per g callus respectively [Table 1]. Moreover, the taro callus was found viable and capable of regenerating plantlets even after 3 years of maintenance in the callus proliferation medium [LSC4], when subcultured at 3–4 weeks interval. Hence, an efficient and fast regeneration system for the in vitro propagation of *C. esculenta* var. *antiquorum* via callus is now available, especially for improvement programs requiring cell suspension and protoplast cultures for genetic manipulation.

Acknowledgement

This research was supported by a grant from the University of Malaya [Vote F–114/82] to S.S.

References

Abo El-Nil MM, Zettler FW [1976] Plant Sci. Lett. 6: 401–8
Basu A, Sethi U, Guha-Mukherjee S [1989] Plant Cell Reports. 8: 333–5
Bradley PM, Fawzi El-Fiki, Giles KL [1984] Plant Sci. Lett. 34: 397–401
Desai HV, Mehta AR [1985] J. Plant Physiology. 119: 45–53
Evans PT, Malmberg RL [1989] Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 235–269
Galston AW, Smith TA [eds] [1985] In: Advances in Agric./Biotechnology Series, Martinus Nijhoff/DR. W. Junk Publishers. Dordrecht, The Netherlands. pp. 3–199
Inoue M, Maeda E [1980 b] Jpn. Soc. Crop Sci. 49: 167–174
Irawati, Webb JK [1984] Annales Bogoriensis 8, 1: 13–22
Ismail El-Hadrami, D'Auzac J [1992] Ann. Bot. 69: 323–325
Ismail El-Hadrami, Housti F, Michaux-Ferriere N, Carron MP, D'Auzac [1993] J. Plant Physiology. 141: 230–233
Kaur-Sawhney R, Tiburcio AF, Galston AW [1988] Planta 173: 282–4
Linsmaier EM and Skoog F [1965] Physiol. Plant 18: 100–127
Malamug JJF, Inden H, Yazawa S, Asahira T [1992 a] J. Jpn. Soc. Hort. Sci. 60 [4]: 935–940
Malamug JJF, Yazawa S, Asahira T [1992 b] J. Jpn. Soc. Hort. Sci. 60 [4]: 927–933
Nyman LP, Gonzales CJ, Arditti J [1983] Ann. Bot. 51: 279–286
Nyman LP, Arditti J [1984] Ann. Bot. 54: 459–466
Sabapathy S, Nair H [1992] Plant Cell Reports. 11: 290–294
Smith TA (1985) Annu. Rev. Plant Physiol. 36: 117–143
Tabor CW, Tabor H [1984] Ann Rev Biochem 53: 749–790
Tiburcio AF, Campos JL, Figueras X, Besford RT [1993] Plant Growth Regulation 12: 331–340
Yam TW, Ichihashi S, Arditti J [1991] Ann. Bot. 67: 317–323