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Influence of *in vitro* preconditioning of citrus microshoots with paclobutrazol on *ex vitro* survival

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The influence of the *in vitro* conditioning and rooting of the microcuttings in different basal media viz., Murashige and Skoog and Gamborg supplemented with various concentrations of paclobutrazol (0.25–4.0 mg/l) on their *in vivo* establishment and growth was investigated using four citrus species viz., *C. reticulata* Blanco (Khasi Mandarin), *C. volkameriana* Ten, and Pasq, *C. reshni* Tanaka (Cleopatra Mandarin), and *C. nobilis* X *C. deliciosa* Tenore (Kinnow). The inclusion of paclobutrazol in the medium had a significant influence on *in vivo* growth especially in relation to height. Paclobutrazol reduced the stem and root growth but at 0.75 and 1.0 mg/l more persistent leaves and new leaves on established plants were recorded. Stomata of leaves cultured in medium supplemented with paclobutrazol showed smaller stomatal apertures. Among the species, Kinnow and *C. volkameriana* showed the maximum leaf area. The optimum concentration of paclobutrazol for maximum establishment was found to be 1 mg/l paclobutrazol (94.6% to 97.2 %) in all the species.

Key words: citrus, culture, paclobutrazol, survival, growth, root, stem, leaves

Introduction

Aseptically produced plants are routinely cultured under high levels of relative humidity leading to a variety of morphological abnormalities, particularly of the stomata and the cuticle, resulting in high mortality rates after transfer of plants to glasshouse or field conditions (GROUT and ASTON 1978). *In vitro* grown plants have reduced development of epicuticular wax resulting in excessive cuticular transpiration on transfer to *ex vitro* conditions (SUTTER and LANGHANS 1982). The inability of stomata to close properly results in further water loss. Improved plant survival rates after transpiration have been promoted by the addition of paclobutrazol to the culture medium (SMITH et al. 1990b). Paclobutrazol causes shortening of the inter nodes of higher plants *in vitro* with additional effects such as a reduction of leaf size, intensification of leaf colour and thickening of roots (GRAEBE 1987). By amending the paclobutrazol level in the culture medium prior to *in vivo* transfer,

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it is therefore possible to improve the quality of established plants derived from aseptic culture. Considering the above, the present study was undertaken to precondition microshoots of citrus plants with different paclobutrazol concentrations and to study the effect on the quality of *in vivo* established platelets and stomatal behavior of *in vitro* derived plants.

Materials and Methods

Terminal microshoots about 1.0–1.5 cm long taken from *in vitro* proliferating cultures of four different citrus species viz., C. *reticulata* Blanco (Khasi Mandarin), *C. volkameriana* Ten. and Pasq. *C. reshni* Tanaka (Cleopatra Mandarin), and *C. nobilis* X *C. deliciosa* Tenore (Kinnow) were aseptically cultured on Murashige and Skoog (MS) and B5 media supplemented with paclobutrazol (0.25, 0.50, 0.75, 1.0 2.0 3.0 and 4.0 mg/L). MS and B5 media without paclobutrazol were used as control. The media contained 0.8% agar, 3% sucrose and pH was adjusted to 5.8 prior to autoclaving. The cultures were incubated at 25 ± 1 °C under a photoperiodic regime of 16 hours light and 8 hours dark at an intensity of 3000 lux at culture level. After conditioning microcuttings with different paclobutrazol levels, rooted microshoots were transferred to culture bottles (450 mL capacity) containing soilrite as a carrier. Bottles were filled up to one third with soilrite saturated with one fourth strength of MS salt solutions before autoclaving at 15 lbs for 20 min. Three to four rooted microshots were planted in each bottle and placed in a culture room for one week. The bottles were shifted to a greenhouse, the caps were loosened gradually and the plants were transferred to a polythene bag containing 1 : 1 soil and well rotten farmyard manure and kept under 75% shade. After four weeks, observations were recorded. Stomatal index was estimated following the method of DHAWAN and BHOJWANI (1987). Stomatal observations on the leaf surface were visualized by cryo-scanning electron microscopy at the Regional Sophisticated Instrumentation Centre following the method of DEY *et al.* (1989) under a scanning electron microscope (JEOL, 35CF, Japan). The data were analyzed as per the procedure of SNEDECOR and COCHRAN (1975).

Results and Discussion

The results of *in vitro* pre conditioning with different basal media supplemented with various concentration of paclobutrazol showed significant variation in the quality of established plants (Tab. 1). Shoot length as well as root length decreased with an increase in paclobutrazol concentration. The number of persistent leaves was low in the controls, while it was at a maximum (3.4) in MS with paclobutrazol at 1.0 mg/L, whereas in B5 medium, it was at its maximum (3.45) at 0.5 mg/L. The production of new leaves was maximum (3.55) in MS with paclobutrazol at 1.0 mg/L, which is on a par with 0.75 mg/L, and minimum leaf number (1.7) was recorded at a paclobutrazol concentration of 4.0 mg/L. Furthermore, plant growth analysis revealed that the inclusion of paclobutrazol in the growth medium inhibited the growth of *in vitro* plants, especially of their height. A decrease in stem elongation is natural, due to the anti-gibberellin activity of paclobutrazol (GRAEBE 1987, RADEMACHER et al. 1984). SMITH et al. (1990a) also found that paclobutrazol had a reverse effect to that of GA_3 biosynthesis. Reduction in plant height with the use of paclobutrazol was also reported by RITCHIE et al. (1991) in chrysanthemum and sugarbeet. KLOCK (1998) also found significant interaction between paclobutrazol concentration and

Tab. 1. Interaction between paclobutrazol and media for various morphological characteristics of *Citrus* species.

Media	Paclobutrazol (mg/L)	Shoot length (cm)	No. of persistent leaves	New leaf No.	Stomatal index	Root length (cm)	Leaf area (cm^2)
MS	0.0	6.05	1.40	3.15	10.89	4.75	6.67
	0.25	4.63	2.90	3.05	10.92	3.04	6.02
	0.5	4.42	3.15	3.25	12.98	2.81	6.48
	0.75	4.45	3.20	3.55	12.18	2.95	5.81
	1.0	3.55	3.40	3.55	11.65	2.88	6.01
	2.0	2.83	3.20	3.30	10.78	2.06	6.24
	3.0	2.68	2.70	2.20	10.67	1.77	5.34
	4.0	2.54	2.60	1.70	10.06	1.59	5.04
B5	0.0	6.58	1.30	2.85	10.75	4.54	6.43
	0.25	3.88	3.20	3.55	12.76	3.23	5.69
	0.5	3.80	3.45	3.70	10.74	2.82	5.68
	0.75	3.61	3.35	3.50	11.49	2.70	5.95
	1.0	3.23	3.35	3.50	11.61	2.44	5.73
	2.0	3.18	3.40	3.10	11.88	2.40	5.38
	3.0	2.85	2.70	1.80	9.71	2.21	5.32
	4.0	2.63	2.45	1.70	9.98	2.23	5.21
CD(0.05)		0.329	0 366	0.383	0.909	0.302	0.389

medium for shoot dry mass, size and height. Paclobutrazol has significant influence on the stomatal index. The inclusion of paclobutrazol in the growth medium also produced smaller stomatal apertures, possibly due to a general reduction in cell expansion caused by paclobutrazol. This is in agreement with the earlier findings of RITCHIE et al. (1991) in chrysanthemum and sugarbeet. There were more persistent leaves in plants treated with paclobutrazol at lower doses. This might be due to the leaves that developed in the culture being retained longer after transplantation, this was contrary to the earlier findings of DONNELLY and VIDAVER (1984), GROUT and ASTON (1978), and GROUT and MILLAM (1985). The number of new leaves formed in the transplanted plants varied, and a maximum number of new leaves were formed in paclobutrazol concentrations of 0.75 and 1.0 mg/L. The number of new leaves formed by a transplant may depend mainly on the number of immature leaf buds formed in the culture, prior to transfer to the *ex vitro* atmosphere. Plants grown in a medium containing the higher levels of paclobutrazol (3 mg/L and above) produced fewer new leaf buds and leaves, and in addition the size of the new leaves was also significantly smaller. After transfer to culture jars, deterioration of persistent leaves was higher from plantlets obtained in the absence of paclobutrazol.

Data pertaining to the morphological response of citrus shoots to paclobutrazol presented in table 2 show significant variations for all the characters studied. The absence of paclobutrazol in the medium resulted in the production of longer shoots. Shoot length decreased proportionately with increasing levels of paclobutrazol in the medium. and the effect was significant even at the lower levels of paclobutrazol, mainly due to reduced stem elongation. Paclobutrazol probably acts both directly, since it is present in auxillary buds, and indirectly via its effect on shoot meristematic activity, decreasing apical dominance which is known to be persistent in plant tissues. Similar findings were reported by BROWNING et al. (1992).

Tab. 2. Morphological response of citrus microshoots to paclobutrazol

Paclobutrazol (mg/L)	Shoot length (cm)	No. of persistent leaves	New leaf No.	Stomatal index	Root length (cm)	Leaf area (cm^2)
0.0	6.32	1.35	3.00	10.82	4.64	6.55
0.25	4.25	3.05	3.30	11.84	3.13	5.85
0.5	4.11	3.30	3.48	11.86	2.81	6.08
0.75	4.03	3.28	3.53	11.84	2.82	5.88
1.0	3.38	3.76	3.53	11.64	2.66	5.87
2.0	3.00	3.30	3.20	11.33	2.23	5.81
3.0	2.76	2.70	2.00	10.19	1.10	5.35
4.0	2.58	2.53	1.70	9.98	1.90	5.12
CD(0.05)	0.223	0.258	0.272	0.643	0.213	0.275

Significant variations were recorded among the species for various morphological characteristics except for shoot length and production of new leaves (Tab. 3). The maximum number of persistent leaves as well as new leaves and maximum leaf area was shown by *C. volkameriana*. Such a variation among the species might be due to the combined influence of paclobutrazol and the medium as well as the genetic nature of the plant material used. A greater number of persistent leaves was recorded in Kinnow followed by *C. volkameriana*. These leaves act as a storage organ or as pseudo-cotyledonary tissues, and encourage the production of new leaves. Kinnow recorded maximum shoot and root growth followed by *C. volkameriana*, though shoot length was statistically insignificant among the species. The *ex vitro* survival per cent recorded with the use of paclobutrazol ranged from 89.2 to 97.2% compared with the range of 78.3% to 85.1% in the control (Tab. 4).

Tab. 3. Mean response of citrus shoots of different species on basal media and Paclobutrazol

Species	Shoot length (cm)	No. of persistent leaves	New leaf No.	Stomatal index	Root length (cm)	Leaf area (cm^2)
KM	3.71	2.78	2.95	10.50	2.73	3.20
KIN	3.89	2.93	2.94	11.19	2.92	8.18
CV	3.84	2.96	3.03	11.30	2.86	8.52
CLM	3.78	2.78	2.95	11.74	2.59	3.34
CD (0.05)	NS	0.183	NS	0.454	0.152	0.194

Tab. 4. *Ex vitro* survival percentage as affected by paclobutrazol in citrus

Species Paclobutrazol (mg/L)	*C. reticulata*	Kinnow	*C. volkameriana*	*C. reshni*
0	79.2	80.5	85.1	78.3
0.25	89.2	89.3	90.3	90.5
ustright 0.5	90.0	93.2	92.9	91.5
0.75	91.2	96.4	93.1	92.4
1.0	96.3	96.7	97.2	94.6
2.0	94.2	90.7	94.3	90.7
3.0	92.1	90.1	92.2	90.4
4.0	90.2	90.3	91.1	89.2

Morphologically, plantlets grown in a medium containing paclobutrazol are short with dark green leaves and short thick roots. The reduced stomatal apertures, increased deposition of epicuticular wax, and thicker roots contributed to the maximum survival. The optimum concentration of paclobutrazol for maximum establishment was found to be 1 mg/L paclobutrazol (94.6% to 97.2 %) in all the species. *C. Volkameriana*, a vigorous species used as rootstock, recorded the highest survival among the species tested.

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