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The induction of hexaploidy in cherry rootstocks using in vitro regeneration techniques

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Summary. An in vitro regeneration technique using root callus has been combined with colchicine application to produce hexaploid plants from the sterile triploid cherry rootstock Colt. Colchicine (50 mg/l) was applied together with auxin (IBA) (3 mg/l) either before or after root primordium formation in single excised micropropagating shoots. In both cases shoots were regenerated in vitro from the putative hexaploid roots. Both methods produced hexaploid plants but more than 70% of the regenerated clones were still triploid. The hexaploid plants were distinguishable from the triploids on morphological as well as cytogenetic grounds. Quantitative characters such as leaf length/breadth ratio, stomatal length and density in the hexaploids were significantly different from both regenerated and non-regenerated triploids.

Key words: Hexaploidy – Cherry rootstocks – In vitro regeneration – Root callus

Introduction

The triploid (2n = 3x = 24) cherry rootstock Colt (*Prunus pseudocerasus*, 2n = 4x = 32 × *P. avium*, 2n = 2x = 16) is of commercial importance since it has a number of desirable horticultural features (Pennel et al. 1983). These include precocious cropping, wide compatibility with both scion cultivars and ornamental *Prunus spp.*, ease of propagation, and the imparting of some degree of dwarfing to the grafted scion.

Colt is, however, only semi-dwarfing and no truly dwarfing rootstock for cherries with the aforementioned features is readily available (Webster 1983). Colt is also a triploid allopolyploid and is thus sterile with no place in future breeding programmes. These features combined with the formulation of in vitro regeneration techniques for *Prunus* cultivars (Druart 1980, Jones et al. 1984) led us to investigate the possibility of coupling colchicine application with adventitious bud regeneration in vitro to produce a fertile hexaploid – possibly with greater dwarfing capacity than its triploid progenitor. Moreover the use of in vitro regeneration procedures that involves a callusing phase often leads to 'somaclonal variation' (Larkin and Scowcroft 1981) increasing the likelihood of variant lines.

The method to be described is based on regeneration from roots or root callus, a means of vegetative propagation established for more than 60 years (Priestley and Swingle 1917).

The use of an in vitro approach ensures more efficient and prolonged contact between colchicine and its target tissues and its presence during de novo shoot or root primordium formation is likely to reduce the probability of chimera formation compared to other methods of application (Broertjes and Van Harten 1978).

This work shows that an in vitro adventitious bud technique can successfully induce polyploidy in a tree species.

Materials and methods

Shoot cultures of the triploid Colt were propagated in vitro as previously described (Jones et al. 1984). Individual shoots were excised and rooted individually in 15 × 2.5 cm test tubes on a Linsmaier and Skoog (1965) basal medium. Two methods were used to generate hexaploids, differing in the timing of colchicine application. In each of the methods approximately 30 replicate micropropagated shoots were used.

Method A

Shoots were rooted in the presence of 3 mg/l indol-3yl-butyric acid (IBA) and 50 mg/l colchicine (Sigma Chem. Co.) for 5 days using a light and temperature regime previously defined by James et al. (1984). Colchicine was added to the medium

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by filter-sterilisation (Millipore 0.22 μm). All shoots were then transferred to a hormone-free medium (James and Thurbon 1979) without colchicine and aventitious roots allowed to emerge for 3 weeks. Complete rooted plants were then transferred to 500 ml screw-topped 'honey jars' containing 1 mg/l 6-benzylaminopurine (BA) and 1 mg/l 1-naphthylacetic acid (1-NAA) and hereafter called 'regeneration medium'. The root system was placed on the surface of the agar with the shoot system to one side. Cultures were grown for 6 to 8 weeks until adventitious shoot regeneration or embryoid formation occurred from the root callus. Regenerated shoots or embryoids were transferred to the same basal medium containing 1 mg/l BA and 0.1 mg/l IBA for shoot proliferation. The micropropagated shoots in turn were rooted in vitro in identical fashion to the original triploid shoots for chromosome counting and for transplantation to vermiculite.

Method B

This method differed only in the timing of application of colchicine; in this case it was not present with the auxin in the initial 5 day period of root primordium formation but instead was present in the hormone-free medium for 3 weeks whilst the adventitious roots emerged. All other conditions were identical to Method A.

Controls

These were of two types:

a) Shoots and embryoids regenerated from root callus in the same way as Methods A and B except that colchicine was not added to any of the media. These controls were given the reference number 8212.
b) Normally micropropagated controls were rooted without any colchicine exposure or regeneration from root callus.

Chromosome preparations

These were made as previously described (James et al. 1985).

Measurements on regenerated plants

After transplanting to greenhouse conditions the leaves of hexaploid and triploid clones were examined for length/breadth ratios, stomatal length and stomatal density. Plants were grown for about 8 weeks after which time they were 19–23 cm tall. Plants of approximately the same height were chosen for leaf and stomatal measurements.

Stomatal measurements

Stomatal lengths were measured using a Stereoscan 100 scanning electron microscope (Cambridge Instruments). Leaves were taken from the fourth expanded leaf below the shoot tip of pot-grown greenhouse trees. Pieces measuring approximately 1 cm^2 were taken from the region half way along the length of the leaf between major veins, and half way between the mid-vein and the leaf margin. These samples were frozen rapidly in nitrogen slush and moved via a Hexland Ltd. cryo-transfer system, where they were sputter coated with gold, to a S.E.M stage cooled to approximately −190 °C. All measurements were taken at zero tilt angle. Stomatal densities were measured according to the method of Beakbane and Majumder (1975).

Statistical treatments

Analyses of variance were performed using a Genstat 5 programme.

Results

Effect of colchicine on adventitious root formation

The adventitious roots that emerged using Method A appeared thicker than those from all other treatments but no measurements were made on them since the entire plantlets were transferred to the regeneration medium. The presence of colchicine did not significantly affect rooting percentage in either method, but when added with IBA (Method A) it halved adventitious root formation irrespective of whether colchicine was present in the hormone-free medium (Table 1).

Effect of colchicine on regeneration from root callus

After 6–8 weeks on regeneration medium roots had callused and a proportion in each of the treatments gave rise to either green shoots directly or to small white heart-shaped embryoids measuring about 1 mm in length and occurring in clumps of six or more. Both green shoots and embryoids could be produced on the same root system and occasionally on the same root in both Methods A and B.

The presence of colchicine with auxin during the period of root primordium formation in the original shoot (Method A) produced less than half the number of regeneration events on a per root basis than its presence without auxin during the period of root growth i.e. Method B (Table 2). Nine per cent of the control root systems regenerated shoots – about midway between the values for the two test methods. No embryoids were formed in control material.

Despite the relatively large number of regeneration events only a proportion of green shoots and embryoids survived to form micropropagating cultures. Losses were mainly due to sudden necrosis of the tissues and this was more apparent in white embryoids than in green shoots. White embryoids that did survive eventually turned green and formed a mass of leaf translucent structures. These cultures eventually took on a more normal appearance and in subsequent cloning of shoots were indistinguishable from shoot cultures derived originally from green shoots.

Several shoots were regenerated from control root systems; these were micropropagated and pooled before chromosome counts (Table 3) were performed on randomly chosen subjects. After several monthly subcultures, each of the somaclones was rooted and chromosome counts performed on the root tip squashes.

Overall one quarter of the clones were shown to be hexaploid but proportionately Method A was better than method B at inducing polyploidy (Table 2).

There was no loss of rooting ability in vitro of any of the plants that had undergone regeneration from

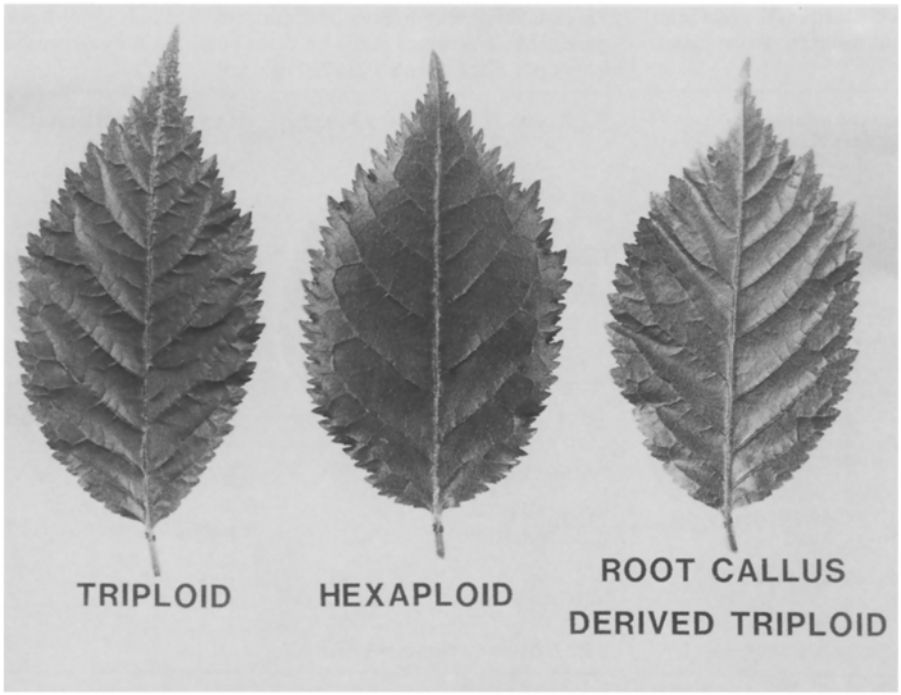

Fig. 1. Appearance of leaves (×0.56) derived from regenerated and control plants after 6 weaks growth under greenhouse conditions. Photographs taken of the fourth youngest leaf from plants approximately 20 cm tall

Table 1. Effect of 50 mg/l colchicine on adventitious rooting

Treatment	Reference	Percentage rooting	Total no. of roots	Roots/ rooted culture
Colchicine present during root initiation	Method A	97	183	7.0 ± 0.9
Colchicine present during root emergence	Method B	87	438	14.1 ± 0.7
Control-colchicine absent	8212	87	412	15.8 ± 1.6

Table 2. The regeneration rate of hexaploids from root callus after colchicine application

	Method A	Method B	Control
No. of plants	27	36	30
No. root systems forming			
a) green shoots	5	21	22
b) embryoids	4	18	0
Total no. sites giving			
a) green shoots	10	41	38
b) embryoids	4	23	0
Total no. of roots	183	438	412
% regeneration[a]	6	13	9
Final no. of clones micropropagated	8	12	1
No. of hexaploids	3	2	0
No. triploid	5	10	1

[a] Number of regeneration sites per root system/root number × 100

callus irrespective of whether they were hexaploid or triploid.

Comparison of vegetative characters in hexaploid and triploid clones

After the initial growth period under glass the regenerated plants whether hexaploid or triploid were less tall than the micropropagated controls. For measurements of leaf sizes and stomatal characters plants of approximately the same size were chosen. The eight youngest fully expanded leaves were used for measurements of length/breadth ratios.

All leaves from hexaploid plants had a significantly reduced length/breadth ratio (Table 4). Morphologically they were also distinct in having a more highly serrated leaf margin and reduced surface relief of the major veins (Fig. 1). They were also more pubescent than the triploids.

Table 3. Chromosome counts from root tip squashes of some of the regenerated, micropropagated and rooted somaclones of the hexaploids and triploids

Clone no.	Regeneration type	Chromosome counts
Method A		
15/2	Green shoots	46, 50, 45, 47, 44, 42, 43, 46, 48, 24, 24, 45, 46, 50, 42, 45, 47, 44, 45, 42
15/3	Green shoots	42, 43, 46, 48, 49, 45
18/2	Green shoots	24, 24, 23, 24
18/3	Green shoots	24, 23, 24, 24, 24, 24, 24, 23, 22, 21, 24, 24, 24, 23, 23, 24, 23, 24, 24, 23, 25, 24, 24
18/8	Green shoots	24, 24, 24, 24, 24, 24, 24, 24, 24, 24, 24, 24
21/1	Green shoots	24, 24
32/1	Green shoots	43, 48, 39, 44, 44, 41, 45, 39, 45, 45, 45, 39, 48, 44, 50, 47, 46
26/1/2	Embryoids	48, 47, 48, 48, 48, 48, 48, 47
26/1/3	Embryoids	48, 48, 46, 48
26/1/4	Embryoids	47, 47
26/1/5	Embryoids	46, 48
26/1/6	Embryoids	48, 43, 48, 43, 46, 48
Method B		
3/1	Green shoots	24, 24, 24, 24, 24
8/1	Green shoots	49, 48, 47, 48, 48, 48, 48
16/1	Green shoots	23, 24, 24, 23, 24
16/2	Green shoots	24, 24, 24, 24, 24, 24
2/1	Embryoids	23, 23, 24, 24, 24, 24
2/3	Embryoids	23
3/4	Embryoids	24, 24, 24, 23
8/2	Embryoids	24, 23, 24, 23, 24, 24
11/1	Embryoids	24, 24, 24, 24, 24, 24, 24
17/2	Embryoids	48, 48, 45, 48, 45, 47
35/2	Embryoids	24, 24
39/3	Embryoids	24, 24, 24, 24

Table 4. Leaf length/breadth ratios in triploids and hexaploids. Measurements made on eight youngest fully expanded leaves per plant. Plants 190–230 mm tall

Plant type	Clone no.	Length/breadth ratio	
		Mean	No. of leaves measured
Hexaploid	15/2	1.75 a	56
	15/3	1.83 a	64
	26/1/2	1.71 a	16
	26/1/3	1.74 a	32
	26/1/4	1.75 a	24
	26/1/5	1.76 a	40
	26/1/6	1.76 a	16
	32/1	1.75 a	24
Normal triploid	CTC	1.95 b	56
Root-callus derived triploid	8212	1.92 b	72
	18/2	2.09 b	24
	18/3	1.93 b	16
	21/1	1.94 b	32
S.E.D. between clones = 0.083			

Values with different suffix letter significantly different at $P = 0.01$ level. S.E. of individual means 0.29–0.40

Table 5. Stomatal length (μm) in 2 triploids and 2 hexaploids. Means and S.E. based on 20 replicates

	Clone	Ploidy	Length (μm)	
			Mean	S.E.
1.	18/2	3x	23.99 a	0.69
2.	2/1	3x	22.68 a	0.42
3.	15/3	6x	34.25 b	0.57
4.	26/1/6	6x	32.08 b	0.63
S.E.D. between clones = 0.83				

Values with same suffix letter not significantly different at $P = 0.01$

Hexaploid leaves had stomata that were about 50% longer than root callus-derived triploids (Table 5) whilst the stomatal density of hexaploid leaves was about half that of either control triploids or root callus derived triploids (Table 6). Although measurements of stomatal lengths of all triploids were of a different order of magnitude from the hexaploids there was a significant difference between triploids regenerated after colchicine exposure and those not so exposed and the normal controls.

Discussion

Colchicine has been used to produce autopolyploids, or more correctly polysomics (Sanford 1983), in a very large number of plant species but nearly always on organised tissues such as axillary buds. The consequence is often the formation of chimeras (Pratt 1983). Broertjes (1974) used an adventitious bud technique to produce a very high percentage of solid non-cyto-chimeral polyploids using detached leaves of herbaceous species. We believe that the work reported here is the first of its kind to combine an in vitro adventitious bud technique with colchicine application for a woody species. This is probably because regeneration techniques for this group of plants are not widely available. These procedures proved more successful than previous attempts on the same material where colchicine was applied to young buds on orchard trees or to meristem tips in vitro (Watkins and Gayner, personal communication). This must be due to the fact that most, if not all, of the stem cells of the primordium are induced to divide in the presence of the alkaloid, whereas applying colchicine to preformed axillary buds is likely to affect only one or two histogenic layers. Adventitious bud techniques must therefore considerably increase the probability of producing homohistont primordia.

Table 6. Stomatal density of hexaploid and triploid plants. Measurements are based on 40 fields of view from the fourth expanded leaf for each clone

Plant type	Clone no.	Density/no. of stomata per unit area of leaf	
		Mean	S.E.
Hexaploid	15/3	11.57 a	0.74
	26/1/2	14.28 b	0.65
	26/1/3	13.10 ab	0.63
	26/1/5	12.75 ab	0.85
	26/1/6	11.62 a	0.80
	32/1	13.53 ab	0.45
Normal triploid	CTC	26.48 c	0.81
Root-callus derived triploid	8212	25.08 c	0.84
Colchicine root-callus derived triploid	18/8	22.23 d	1.03
S.E.D. between clones = 0.80			

Values with different suffix letter significantly different at $P = 0.01$

Theoretically the two methods used here should achieve the same result since colchicine is present throughout the period of shoot or root primordium formation. In method A shoot regeneration occurs from putative hexaploid roots whilst in method B they regenerate from triploid roots in the presence of colchicine. The first method appears to be more efficient but this is offset by the detrimental effects of colchicine on root formation. In an easily-rooting subject this may be of little consequence, but where the subject is difficult to root in vitro, method B may be the method of choice. Despite the relative success of both methods diplontic selection obviously still occurs since the majority of the regenerated shoots are triploid despite prolonged exposure and contact with colchicine. However, the convenience of the method and the obvious advantage of being able to micropropagate rapidly the regenerated hexaploids and mutant triploids should make in vitro adventitious bud techniques the method of choice for polyploid production in the future. Moreover the ability to regenerate from leaf discs in fruit tree tissues (James et al. 1984) may replace the need to use roots since leaves are more abundant and more convenient to use when micropropagation systems are available.

Chromosomes in cherry are very small (usually less than 1 μm in length) which hinders cytological analysis particularly at the higher ploidy levels. This may account for the unevenness of the counts in the hexaploids, although any procedure involving a callus phase is likely to produce plants with cytogenetic abnormalities (Karp et al. 1982). Nevertheless only clone 15/2 appears to be obviously chimeral and we assume that the others are either hexaploids, triploids or aneuploid derivatives of them. The possibility that some of the plants may be chimeras cannot be assessed fully until flowering occurs in a few years' time. Only then will it be possible to look for meiotic irregularities in the gametophytic tissue, measure pollen size, and compare the ploidy of the L2 layer with that of the root tip (L3).

Several reports from various crop plants show that colchicine treatment induces aneuploid (Ahloowalia 1967; Bergner et al. 1940; Hermsen et al. 1970) and euploid mutants (Franzke and Ross 1952; Gilbert and Patterson 1965; Pandey 1968) as well as the desired autopolyploid (Lapins 1975). Thus, variants may have been produced by both colchicine treatment and somaclonal variation. There were, however, no obvious differences in leaf and stem morphology within the triploid and within the hexaploid populations when grown under greenhouse conditions. Nevertheless, all triploids regenerated with and without colchicine treatment are being field tested.

Perhaps of greater significance than individual chromosome counts is the good correlation between ploidy and stomatal characters where the representative hexaploids had much larger stomata and correspondingly reduced density per unit area than the triploids. In apple Beakbane and Majumder (1975) showed that the more dwarfing rootstocks had a lower stomatal density than their more invigorating counterparts although in this case all rootstocks were diploid.

One of the aims of this work was to produce a rootstock with more dwarfing characteristics than the original triploid. Though it is too early for growth trials to confirm whether this has been achieved, past work gives us reasons for optimism. Tal (1980) described the effects of changes in ploidy levels on transpiration rate which suggests that the hexaploids may have lower transpiration rates and higher root pressure than the triploids. In addition, the nucleotypic theory of Bennett (1972) suggests that cell DNA content and developmental rates are inversely related. Any decreased growth in our hexaploid material would only be of benefit, of course, if it also decreased the growth of a scion variety grafted onto it. Such horticultural advantage however may be offset by increased drought susceptibility since it is well known that cell size and drought tolerance are inversely related (Iljin 1957). The triploid Colt already has a tendency to be drought susceptible and so the larger cell size of the hexaploid may set it at a further disadvantage.

The effects of ploidy doubling on plant phenotype are well known and include changes in vegetative characters such as reduced height, thicker, darker green leaves with a more highly serrated margin, increased thickening of petiole and stem with correspondingly shortened nodes and wider crotch angles, features that have all been noted in fruit trees (Sanford 1983) and other plants (Mathew and Thomson 1984). The hexa-

ploid plants produced in this work were distinguishable from the triploids even at the tissue culture stage on the basis of darker foliage and more serrated leaf margins.

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