A review of factors influencing the genetic stability of micropropagated bananas.

M.K. SMITH*

A REVIEW OF FACTORS INFLUENCING THE GENETIC STABILITY OF MICROPROPAGATED BANANAS.
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ABSTRACT - The occurrence of off-types (somaclonal variants) from micropropagated bananas is of concern to the banana industry throughout the world. Somaclonal variation is influenced by both intrinsic factors, such as the genetic stability of the cultivar or genotype being micropropagated, and extrinsic or culture-induced factors. Genetic changes induced during the process of tissue culture can be influenced by the choice of explant, the choice of culture medium (particularly the nature and concentration of phytohormones), the period spent in culture and the degree of dedifferentiation the tissues undergo in culture. Strategies for minimizing somaclonal variation from micropropagated bananas are proposed.

UNE REVUE DES FACTEURS INFLUENÇANT LA STABILITE GENETIQUE DES BANANIERS ISSUS DE MICROPROPAGATION.
M.K. SMITH.
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RESUME - L'apparition de variants somaclonaux parmi des bananiers obtenus de micropropagation intéresse la production de cette espèce dans le monde entier. La variation somaclonale est influencée tant par des facteurs intrinsèques tels que la stabilité génétique du cultivar ou le génotype que par d'autres, extrinsèques liés à la culture. Les modifications génétiques peuvent dépendre du choix de l'explant, du milieu de culture (nature et concentration en phytohormones), de la durée de celle-ci et du degré de dédifférenciation des tissus. On propose des méthodes pour réduire la variation somaclonale.

INTRODUCTION

The technique for the establishment of banana plants from excised shoot tips was first reported by Ma and Shii (1972) and has been further modified by Hwang *et al.* (1984) and Cronauer and Krikorian (1984) for the rapid *in vitro* propagation of bananas. One of the major features of the technique, as it is now used, is that multiplication can be induced by releasing dormant buds at the leaf bases of the explants. Subculture from the proliferating mass of shoots which results ensures a steady and rapid rate of increase.

Commercial laboratories are currently producing banana plants using *in vitro* propagation techniques for industry plantations in Taiwan, Jamaica, Israel, South Africa and Australia. With the field establishment of these plants, reports of a large percentage of off-types (variants) in the population have surfaced. Variability has ranged from 3% in Taiwan (Hwang and Ko, 1986), 9% in Israel (Reuveni *et al.*, 1984), 21% in Australia (Kebby, 1987) to 25% in Jamaica (Stover, 1986). Table 1 lists the off-types that have been observed in Australia and overseas. Dwarfism is by far the most common off-type observed amongst Cavendish clones.

This high incidence of off-types from micropropagated bananas is of concern to the industry, not only in Australia but worldwide. This review attempts to identify those factors that may be influencing the variation observed in micropropagated bananas and outlines some of the steps that are being taken by the Australian industry to minimize this variation. Areas in need of further research are indicated. Particular attention is given to the problem of dwarf off-types.

FACTORS INFLUENCING SOMACLONAL VARIATION

It is now firmly established that genetic changes can occur during the process of tissue and cell culture. Many of these changes are 'locked' into the genome of the regenerated plants and can therefore be transferred to successive generations. This phenomenon, called somaclonal variation,

* - Research Horticulturist - Department of Primary Industries Maroochy Horticultural Research Station - P.O. Box 5083 - Sunshine Coast Mail Centre Nambour, Qld, Australia, 4560.

TABLE 1 - Somaclonal variants encountered from micropropagated Cavendish clones.

Stature	a.	Various degrees of dwarfism. Choking sometimes occurs when throwing a bunch. The peduncle is much shorter and hands packed much closer to each other on the stem than normal.
	b.	Miniature plants with thin pseudostems, fewer hands and long tapering bunches.
	c.	Giantism. Excessively tall plants with long distance between the point of leaf emergence.
Foliage.	a.	Narrow drooping leaves ; characteristic of tetraploids (an extra set of chromosomes).
	b.	Variegated leaves (shades of yellow and pale green) sometimes resembling «mosaic».
	c.	Waffled or wavy edges of leaf with changes in leaf thickness.
	d.	Increased waxiness.
Pseudostem.	a.	The pseudostem, petiole and midrib turns black after bunch emergence.
	b.	Purple-black pseudostem.
	c.	Greenish pseudostem, petioles and midribs.
Bunch.	a.	Small bunches with short fingers. The leaves are dark pigmented and droop over.
	b.	Hairy fruit.
	c.	Narrow and elongate male bud.

can be defined as genetic variability generated during tissue culture (Larkin and Scowcroft, 1981).

This variation can involve point mutations, at one end of the spectrum, to gross ploidy changes at the other end. Variability is most likely the result of both intrinsic factors, such as the genetic stability of the particular cultivar or genotype under investigation, and genetic alterations induced during the process of tissue culture (Ammirato *et al.*, 1984). Scowcroft (1984) and George and Sherrington (1984), after reviewing the available literature on genetic variation in plants propagated through tissue culture, summarized the following factors as being known to influence the level of somaclonal variation observed in plants :

1) If callus formation is a significant phase in the propagation cycle, then it can be expected that plants will show a higher level of somaclonal variation than those that do not undergo an intervening callus phase of growth.

2) The frequency of somaclonal variants among plants propagated through tissue culture increases with prolongation of the culture period.

3) Asexually-propagated species can be expected to display a higher frequency of somaclonal variation than seed-propagated species.

4) Some genotypes are more prone to genetic instability than others and this can be highlighted during tissue culture propagation.

5) The composition of the culture medium, particularly the nature and concentration of plant growth regulators used in the medium, may lead to genetic changes in tissue culture-propagated plants.

Taking each of these main points in turn :

Culture Mode.

Scowcroft (1984) has ranked tissue culture systems in order from low to high for genetic instability as follows: micropropagation from isolated buds and meristems, adventitious shooting, somatic embryogenesis and organogenesis from callus, cells and cultured protoplasts. Because banana micropropagation involves the release of dormant buds at the leaf bases of the explants, then callus formation does not occur under the culture conditions outlined by Hwang *et al.* (1984) and Cronauer and Krikorian (1984). Should commercial laboratories use a combination of phytohormones that encourage a dedifferentiated callus growth phase prior to shoot multiplication, then somaclonal variation may be enhanced.

Micropropagation from isolated buds and meristems would provide the best option to minimize somaclonal variation and this is the recommended system used in propagating bananas in culture. However variation in micropropagated plants does occur. Swartz *et al.* (1981), using rapid propagation from stolon meristem tips of strawberry, evaluated some 500 plants from each of three cultivars. Variant plants were found including runnerless and female-sterile types and those with compact trusses. Dwarf variants comprised 1.2% of the total population.

Reports on plants produced by micropropagation rarely report the actual level of variation. Defined research is needed to establish the level of variation in micropropagation plants in comparison to that found as a consequence of somatic embryogenesis and organogenesis from callus.

Period spent in Culture.

Prolonged periods of tissue culture are known to result in an increased frequency of gross chromosomal aberrations (Meins, 1983). Also the frequency of somaclonal variants among plants regenerated from tissue culture also increases with length of time in culture, being more of a problem in plants regenerated from callus and cell cultures than in micropropagated plants.

In addition, if a variant should arise and go undetected through successive subcultures, the impact of a single off-type is magnified. Therefore if a dwarf variant arises early in the culture cycle then subsequent subcultures will increases its numbers significantly. Hence the culture technique as well as genetic change contributes to the problem. Should the variant multiply faster but not be readily identified the culture technique becomes the dominant factor.

Sexual versus Asexual Species.

With seed development, in sexually-reproducing species, the normal processes of meiosis and fertilization will eliminate chromosomal abnormalities in favour of those gametes with the 'normal' chromosome complement. Banana, being a sterile triploid, is able to conserve its unique genome only through asexual vegetative propagation. Occasionally 'sports' or off-types arise naturally. The process of micropropagation apparently increases the frequency of these off-type events.

Genotype.

Limited evidence indicates that the genotype of the mother plant has a significant effect on the extent of variation generated during culture. In strawberry, cultivar differences occur in the frequency of off-type plants (Swartz *et al.*, 1981). Among plants derived by adventitious shoot formation from leaf explants of *Begonia* x *hiemalis*, Roest *et al.* (1981) found that in one cultivar 43% of regenerante were variant (colour, size and form of leaves and flowers) whereas for another cultivar only 7% were variant.

A similar problem may exist with banana cultivars as some genotypes have yielded a higher percentage of dwarf off-types than other genotypes (Table 2). More research is needed to help resolve this issue as multiplication from genetically stable genotypes would be preferred.

Media Composition.

Scowcroft (1984) states that tissue culture media and growth regulators appear not to be mutagenic *per se*. He bases this on the mutagenic assay using the *Tradescantia* stamen hair system developed by Grant and Zura (1982) which has been developed as a sensitive system for studying somatic mutations resulting from ionizing radiation or chemical mutagenesis. Dolezel and Novak (1984) tested for the mutagenic effect of a wide range of phytohormones and found no somatic mutation rate significantly greater than the spontaneous rate.

It can be argued that if a particular hormone induces dedifferentiation of plant tissues, then it is the dedifferentiation process and its maintenance that can cause genetic instability and not the hormone itself. Similarly the culture medium may act to increase the proportion of genetically abnormal cells that appear or are already present in an explant by differentially influencing their rate of division.

George and Sherrington (1984) state that the use of high concentrations of auxins and cytokinins in culture media can result in plants being morphologically different from normal plants. In most cases these involve physiological or epigenetic changes that are reversible. In other words the plants can 'grow out' of their particular abnormality after being transfered to soil. In some cases, however, cytokinins have been implicated in causing genetic changes (George and Sherrington, 1984). A better understanding of the causes of aberration would be useful in developing strategies for circumventing them. For example, if a connection between high levels of synthetic growth regulators and the appearance of phenotypic aberrations can be established then modifications of the types of growth regulators used can be instituted.

STRATEGIES FOR MINIMIZING SOMACLONAL VARIATION IN MICROPROPAGATED BANANAS

Having examined the factors that can influence variation in micropropagated bananas, it is useful to identify the following possible strategies to minimize this variation.

1) Commercial laboratories involved with micropropagating bananas for the industry should be made aware of the potential incidence of, and the factors that influence somaclonal variation. Advice would be given of the methods of identifying high risk growth characteristics *in vitro* and strategies for minimizing these problems. Such 'feedback' should modify work practices that may

TABLE 2 - Tissue culture planting survey ; July 1986. The number and percentage of dwarf off-types by clone.

Cavendish cv. Williams Clone	Number of Plants	Number of Off-types	% Off-types
C3	1068	339	31.7
C2	211	13	6.2
C5	336	14	4.1
C7	414	67	16.0
C26	101	11	10.9
Total	2130	444	20.8

be contributing to excessive off-types occuring in commercial plant batches.

2) Because the culture medium, particularly the nature and concentrations of phytohormones used in the culture medium, may contribute to the production of off-types it is necessary to determine a lower limit of phytohormones concentration where multiplication is possible but the production of off-types is minimized. The use of 5 mg l^{-1} BAP (benzyl amino purine) for shoot multiplication is widely used (Cronauer and Krikorian, 1984 ; Jarret *et al.*, 1985 ; Gupta, 1986) however good multiplication is also possible for a range of cultivars at half this concentration (ie. 2.5 mg l^{-1} BAP, Wong, 1986). Further research is needed in this area. In the meantime BAP concentrations in the range of 2-2.5 mg l^{-1} is recommended for banana multiplication.

   The use of any combination of phytohormones that cause the tissues to dedifferentiation to form callus should be discontinued.

3) It has been suggested that cultures initiated from floral apices (bells) show a greater percentage of off-types than from sucker-derived apices. The basis of this concern is commercial experience in Australia, but no comparative testing under controlled experimental conditions has been done. Until such work is done it is recommended that cultures be initiated from vegetative apices of suckers and that bell-derived plants should be omitted from collections geared towards multiplication of plants for the industry.

4) It is important to remember that both the frequency of genetic events leading to off-types and the proportion of off-types multiplied in culture will increase with time.

   Hwang (pers.comm) states that they have been successful in limiting the incidence of off-types to 3% and this is attributable, in part, to limiting multiplication to no more than 20 000 plants per initial explant. If more plants are to be produced then a proportionate number of suckers are necessary from which to initiate lines. Reuveni (pers comm) takes a more conservative approach and says the limit should be no more than 1 000 plants per initial explant. Clearly some work is necessary to establish the upper limits and the cooperation of commercial laboratories would be invaluable in making this sort of assessment possible.

   Commercial laboratories may wish to consider a programme where important clones are regularly re-initiated from suckers to ensure genetic uniformity. Alternatively the laboratories may wish to consider a programme where important clones are maintained in a slow-multiplication cycle (ie. low to no phytohormones) to keep a healthy stock of material to draw upon for periods of rapid increase. At this point in time the former approach is recommended and the latter is a goal to aim for once a greater understanding of the incidence of off-types has developed.

5) The occurrence of off-types in the field should be carefully monitored. Good records are also necessary at all stages of the multiplication, hardening-off and field establishment process. It is important to be able to trace the origin of the plant nursery through to the tissue culture laboratory for each specific planting. Coupled with this must be the ability to trace batches of plants and their history of treatment through each process by well kept and standardised records. This should help to identify any problem areas and to remedy the situations which arise efficiently.

6) Commercial laboratories should rogue any off-type plants at the culture level and nurseries should rogue prior to field planting. Only morphologically normal plants should be permitted to be planted in the field. This will eliminate many of the thin-leaved off-types, variegated leaf off-types and other morphologically aberrant plants.

   Dwarfs will continue to be a problem in Cavendish clones until a more reliable screening and selection procedure is found and implemented. The off-type problem in Australia can largely be attributed to the fact that little or no selection was practised at either the culture or nursery level. Dwarfs which are difficult to separate from normal plants at a young age mostly went undetected and showed themselves in the field just prior to bunch emergence.

   Taiwanese experience suggests that subtle differences in leaf and pseudostem morphology can be selectively applied at the nursery level to rogue dwarf off-types (Hwang, pers comm). A trial is currently underway to define and determine if morphological markers can be used in the Williams cultivar to distinguish dwarfs from normal plants under the conditions existing at QBan nurseries (industry-certified). Selected and normal plants require field evaluation to determine if a roguing technique based on morphology has any merit under Australian conditions.

   Recent work by Reuveni and colleagues in Israel suggests that biochemical markers may be used to distinguish dwarf from normal plants at the culture level. Studies of this nature may have increasing relevance in the absence of a system based on morphological markers.

   Until a reliable screening and selection programme is developed, dwarf off-types will continue to be observed in field plantings.

   Dwarf off-types represent both a problem and a challenge. A major problem is the long lag-time from culture initiation to final detection of many off-types in the field. A time frame that involves several years. The above guidelines represent some of the strategies for minimizing the problem. The challenge lies in both developing suitable cultural practices to minimize the formation of dwarf off-types and in developing appropriate screening and selection techniques to detect dwarf off-types as early in the propagation chain as possible, and definitely before they reach the field. The above guidelines can be implemented, however several years of further research and development are necessary to improve culture and off-type screening

techniques to establish at what level of confidence a commercial banana tissue culture propagation scheme can operate.

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ÜBERSICHT DER EINFLUSSFAKTOREN DER GENETISCHEN STABILITÄT VON BANANENPFLANZEN, DIE DER MIKROSKOPISCHEN VERMEHRUNG ENTSTAMMEN.

M.K. SMITH.

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KURZFASSUNG - Die im Wege der mikroskopischen Vermehrung gezüchteten, somaklonalen Varianten der Banane sind für die Produktion dieser Spezies in der ganzen Welt von Interesse. Die somaklonale Variation wird von endogenen Faktoren wie genetische Stabilität der Zuchtsorte bzw. des Genotyps, sowie von exogenen, anbaurelevanten Parametern beeinflusst. Für die genetischen Modifikationen mögen folgende Gesichtspunkte bestimmend sein : Wahl des Explantats, Zuchtmedium (Natur und Konzentration an Pflanzenwuchsstoffen), Zuchtdauer und Entdifferenzierungsgrad der Gewebe. Zur Reduzierung der somaklonalen Variation werden einschlägige Methoden vorgelegt.

UNA REVISTA DE LOS FACTORES QUE INFLUYEN EN LA ESTABILIDAD GENETICA DE LOS BANANOS PROCEDENTES DE MICROPROPAGACION.

M.K. SMITH

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RESUMEN - La aparición de variantes somaclonales entre bananos obtenidos de micropropagación interesa a la producción de esta especies en el mundo entero. La variación somaclonal esta influenciada tanto por factores intrínsecos como la estabilidad genética del cultivar o el genotipo como por otros, extrínsecos ligados al cultivo. Las modificaciones genéticas pueden depender de la elección del explante, del medio de cultivo (naturaleza y concentración en fitohormonas), de la duración de éste y del grado de diferenciación de los tejidos. Se proponen métodos para reducir la variación somaclonal.