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Herbicide-tolerant tobacco mutants selected *in situ* and recovered via regeneration from cell culture

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SUMMARY

The herbicides Bentazone and Phenmedipharm kill the leaves of intact tobacco plants but do not affect callus cultures. Tolerant mutants were isolated by treating leaves of previously γ-irradiated haploid plants with herbicide then excising and culturing the green herbicide-resistant cell clones on the otherwise yellowed leaves. Among plants subsequently regenerated were a total of ten stable independently isolated mutants. Sexual crosses show these ten represent four Bentazone and two Phenmedipharm loci; all mutants were recessive to wild type.

1. INTRODUCTION

Recent technical advances in plant tissue culture and somatic cell genetics have raised the hope that microbiological techniques could be used to isolate useful mutants with greater efficiency than traditional whole plant screening methods (Carlson & Polacco, 1975; Day, 1977; Rice & Carlson, 1975). The most developed model system for this approach exists in tobacco, where (1) anther culture produces haploid plants, (2) tissues can be grown *in vitro* as callus for many cell generations and (3) cells in culture can be induced to regenerate into new plants. This technology already permits the selection and recovery of new plant varieties after altering the genetic information of cultured cells by such means as mutagenesis (Carlson, 1973) and protoplast fusion (Carlson, Smith & Dearing, 1972).

A limitation of this system lies in the nature of plant cells in culture. Although cells from several parts of the plant (for example, meristem, leaves and roots) can readily be cultured, the resulting callus invariably exhibits a profound change involving the loss of many properties which uniquely characterized the differentiated parental tissue. This loss severely constrains the advantages to be gained from a tissue culture system especially when the objective centres on studying the biochemical, genetic or agronomic properties of the intact developing plant (Day *et al.* 1977). Clearly, a combination of whole plant and cell culture techniques would aid investigations of plant functions expressed solely in differentiated tissues.

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This report describes a method for direct *in situ* selection of variants which alter the genetically determined characteristics of primary plant tissues. Young haploid plants are mutagenized and then allowed subsequent growth and cell division to increase the size of induced mutant clones in the soma. The tissue of interest is then subjected to a selection or screening regime; cell clones exhibiting the desired phenotype are excised, placed in tissue culture and immediately induced to regenerate whole plants. The whole plant is then tested for continued expression of the desired phenotype. Subsequent diploidization permits sexual crossing and genetic analysis of the mutant character.

2. MATERIAL AND METHODS

The herbicides used in this study were Bentazone (3-isopropyl-1H-2,1,3 benzothiadiazine-4-(3H)-one 2,2-dioxide) obtained from Base Aktiengesellschaff and Phenmedipharm (methyl *m*-hydroxycarbonilate *m*-methylcarbonilate) produced by NOR-AM Agricultural Products, Inc.

Haploid plants and tissue cultures were obtained by anther culture of *Nicotiana tabacum* var. 'Wisconsin 38'. All tobacco plants were grown under standard greenhouse conditions. Tissue culture media utilized the Linsmaier & Skoog (1965) formulation. Medium for callus growth contained 3 mg/l. indole-3-acetic acid (IAA) and 0·3 mg/l. kinetin. Regeneration medium contained 0·3 mg/l. IAA and 10 mg/l. N_6-(2-isopentenyl)-adenine.

Mutagenesis was performed by irradiating plants with 500 rad of γ-rays from a ^{60}Co source.

3. RESULTS AND DISCUSSION

We attempted to isolate mutants of tobacco exhibiting resistance toward two normally lethal herbicides: Bentazone and Phenmedipharm. Both compounds characteristically kill tobacco, causing the leaves to turn yellow and die. When small expanding leaves (less than 2 cm in length) of control plants were sprayed with 0·1 ml of Bentazone at 0·03 mg/ml. or 0·1 ml. of Phenmedipharm at 0·1 mg/ml., their expansion was reduced to about one-third normal size and their colour changed to a uniform yellow. No green sectors appeared in 35 sprayed control leaves. Tissue excised from these yellow leaves failed to proliferate when placed on callus culture medium. Callus from untreated leaves grew normally on the same medium. In contrast to whole plant reactions, neither herbicide (in concentrations up to 0·3 mg/ml. of culture medium) affected growth rate or morphology of preestablished callus cultures. Herbicide reactivity therefore reliably distinguished primary leaf tissue and undifferentiated callus.

Herbicide-resistant isolates were obtained as follows. A summary of the procedure appears in Table 1. Forty young haploid plants were mutagenized by treatment with γ-rays. After further growth and cell division occurred, about ten immature leaves on each of twenty irradiated plants were sprayed for each herbicide (as described above). Upon continued growth and leaf expansion, many of the otherwise yellowed leaves retained small green islands of tissue resembling those

Table 1. *Sequence of operations for isolating herbicide tolerant mutants in tobacco*

(The number of putative mutants surviving each step is illustrated.)

Mutant isolation step	Bentazone tolerance		Phenmedipharm tolerance	
	Survivors	%	Survivors	%
1. Irradiate haploid plantlets and allow subsequent cell division	(20 plants)*		(20 plants)*	
2. Treat leaves with herbicide	(200 leaves)*		(200 leaves)*	
3. Excise and culture green clones	78	100	66	100
4. Stimulate plant regeneration	71	91·0	52	78·8
5. Test plants for herbicide resistance†	15	19·2	7	10·6
6. Diploidize resistant plants	15	19·2	7	10·6
7. Analyse genetically via sexual crosses	8	10·3	2	3·0

* The number of plants or leaves utilized in each of the two initial steps was chosen arbitrarily.

† The quantities and concentrations of herbicide solutions sprayed on to leaves of test plants were identical to those used for the selection procedures described in the text. Leaves so treated turned yellowish green in resistant plants and full yellow in sensitive plants. The easily distinguishable mutant phenotype is similar to that observed in tobacco strains heterozygous for the sulfer, *Su*, mutation previously described by Burk & Menser (1964).

Table 2. *F_2 segregation of Bentazone (B) and Phenmedipharm (P) resistant mutants*

Mutant	F_2 segregation (100 progeny)		Suspected ratio	χ^{2*}
	Sensitive	Resistant		
B-1	91	9	15:1	0·86
B-2	79	21	3:1	0·65
B-3	67	33	3:1	3·00
B-4	88	12	15:1	4·70
B-5	71	29	3:1	0·65
B-6	96	4	15:1	0·53
B-7	81	19	3:1	1·58
B-8	80	20	3:1	1·08
P-1	77	23	3:1	0·12
P-2	84	16	3:1	3·85

* Significant at 5% level if $\geqslant 3{\cdot}84$.

often seen on mosaic virus infected leaves (Atkinson & Mathews, 1970). These green sectors were carefully excised and placed in regeneration culture medium. Whole plants eventually grew from most of the cultured sectors with 21·1% Bentazone and 13·5% Phenmedipharm originated plants retaining resistance to their respective herbicides.

Genetic analysis of the new herbicide resistant varieties was done by back-crossing to wild type after colchicine induced diploidization. The F_1 progeny invariably lost the resistant phenotype demonstrating the recessive nature of the mutants. Herbicide resistance was recovered in the F_2 progeny from only eight of

Table 3. *Genetic complementation between herbicide* resistant mutants*

Complementation group	Mutant(s)
B I	B-1, 3, 4, 6
B II	B-2, 7
B III	B-5
B IV	B-8
P I	P-1
P II	P-2

* Bentazone (B) and Phenmedipharm (P).

the 15 Bentazone and two of the seven Phenmedipharm isolates. The F_2 segregations are given in Table 2. Failure of the remaining putative tolerant isolates to reappear among the F_2 progeny might be due to genetic or epigenetic instability of the original lesions. Cytoplasmic genes also would be unlikely to segregate in the F_2 generation thus producing the same result. However, we have yet to obtain the experimental data necessary to decide among these possibilities. Of the ten stable F_2 mutants, seven behaved as expected for single gene mutants (Table 2), while the F_2 segregations in the remaining mutants (B-1, 4, 6) behaved as if the trait were due to two independently segregating genes.

Complementation between the mutants was analysed by making all possible pairwise crosses in order to see if progeny would be sensitive or resistant. The results of these tests (Table 3) demonstrated that mutants B-1, 3, 4 and 6 are allelic while B-2 and 7 belong to a second complementation group. Allelism did not appear among the other mutant combinations. The finding that B-1, 4 and 6 each segregated as if they were two genes and also were allelic to one another and the single gene isolate, B-3, is unexpected. Such a result could have occurred if the three mutants were unstable alleles of a single genetic locus with B-3 being a stable form of the same gene. The accumulation of revertants during growth of the F_1 plants could produce the *appearance* of double mutants in the F_2 segregations. Alternatively, the 'two genes' explanation might be reasonable if the original parental strain possessed one of the mutant loci as a 'cryptic' gene. However, the actual genetic explanation is not yet clear.

We expect that *in situ* mutant selection followed by *in vitro* recovery will be applicable to a variety of mutant types and plant systems. The easiest applications should be genotypes conferring tissue-specific resistance to toxic or lethal agents such as pollutants, pathotoxins and pathogenic micro-organisms. In theory any type of tissue ought to be a suitable selection target provided that regeneration capacity exists. In some cases, cultural organs might prove more suitable than intact plants; as one example, in selecting tolerance by roots to soil-borne toxic agents.

The application of *in situ* selection is of course limited to plant systems for which the regeneration can be accomplished from primary cultures. It is important to note that the *in situ* method does not depend on long-term cell culture and uses

immediate regeneration of the selected cell clones; a considerable number of agronomically important plants can be so regenerated from primary explants (Reinert & Bajaj, 1977).

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