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In vitro selection for 2,4-D tolerance in red clover *

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Summary. In vitro, selection is a viable method of selecting herbicide-tolerant crops. This research was to evaluate in vitro selection techniques for enhancing 2,4-D [(2,4-dichlorophenoxy) acetic acid] tolerance in red clover (*Trifolium pratense* L.). In vivo and in vitro responses to 2,4-D of eight diverse red clover populations were correlated ($r = 0.77$), justifying in vitro selection for 2,4-D tolerance. Suspension cultures of a red clover genotype capable of regeneration were plated onto agar-based nutrient media supplemented with 0.18 m*M* 2,4-D for selection experiments. After two cycles of selection, 16 2,4-D tolerant callus lines were identified based on visual growth assessment. These lines were evaluated for 2,4-D tolerance (based on 2,4-D content), using a 2,4-D bioassay procedure which consisted of placing selected callus tissue pieces on top of oat (*Avena sativa* L.) coleoptile or internode sections. The relative amount of 2,4-D in the callus tissue was estimated by the amount of oat section elongation after 24 h. Two of the more tolerant callus lines had 61% and 83% less 2,4-D in their tissues than the susceptible control tissue. These studies indicated that in vitro selection can enhance the levels of 2,4-D tolerance in red clover callus tissue.

Key words: *Trifolium pratense* L. – Tissue culture – Herbicide tolerance – Phenoxy herbicides – Callus

Introduction

Herbicide tolerance (i.e., the ability to continue growth when exposed to normally toxic concentrations) has been identified as one of the traits that is well-suited to in vitro selection schemes. Using these methods, millions of potentially different cells can be quickly and efficiently screened. However, in most systems these cells trace to one or a few different genotypes, from which it is hoped that either spontaneous or induced mutations may be recovered by the high selection pressure. Cell culture conditions can provide new sources of crop variability through somaclonal variation and produce plants with alternate forms of herbicide tolerance (Hughes 1984). Callus tissue cultures may also provide a unique system to study the biochemical and physiological aspects of plant-herbicide interactions (Chaleff and Mauvais 1984).

In vitro selection for 2,4-D [(2,4-dichlorophenoxy) acetic acid] tolerance has been attempted by several researchers with only modest success (Swanson and Tomes 1980, 1983). This may be attributed to the complex nature of the mode of action of 2,4-D, and to the complicated genetics that are probably required to obtain tolerance. A tolerance mechanism could operate either through an alteration at the site of action or an alteration of an enzyme system involved in 2,4-D metabolism. Successful in vitro selections for herbicide tolerance have involved tolerances that are under the control of qualitative genetic systems, such as that shown for picloram (4-amino-3,5,6-trichloro-2-pyridine carboxylic acid) tolerance in tobacco (*Nicotiana tabacum* L.) (Chaleff and Parsons 1978). As an auxin, the herbicide 2,4-D may induce changes in callus tissue that may alter regenerative potential. These factors may contribute to the difficulties involved in selecting for 2,4-D tolerance in vitro.

Swanson and Tomes (1979, 1983) isolated callus of birdsfoot trefoil (*Lotus corniculatus* L.) that was capable of growth on high levels of 2,4-D. However, the three tolerant callus lines produced regenerated plants with low levels of tolerance to 2,4-D. The regenerated plants

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did not express as much tolerance as the tolerant control, which was selected by five cycles of recurrent selection at the whole plant level. Ono (1979) selected a stable variant cell line of tobacco that was tolerant to 2,4-D. Regenerated plants did not express the 2,4-D tolerance but callus re-induced from the regenerated plants retained the 2,4-D tolerance in vitro. These calli also possessed cross-tolerance to the auxins IAA, NAA and picloram (Nakamura et al. 1985). Gressel (1979) isolated a strain of carrot (*Daucus carota* L. var. *sativa* DC.) cells which tolerated a concentration of 2,4-D 100 times that of the unselected control. After 1 year of growing the cells in the absence of 2,4-D, these cell lines retained their tolerance. Regeneration of tolerant lines was not reported. Oswald et al. (1977) conducted experiments on 2,4-D tolerance in white clover (*Trifolium repens* L.) suspension cultures. They found that a 5-day pretreatment with either 2,4-D, 2,4,5-T [(2,4,5-trichloro-phenoxy) acetic acid], or 2,4-DB [4-(2,4-dichloro-phenoxy) butanoic acid] increased cell tolerance levels to these herbicides. Tolerance was maintained in succeeding cell generations but no plant regeneration was reported.

The feasibility of an in vitro selection system increases if whole plant and in vitro response are correlated (Gressel et al. 1978). The fact that 2,4-D tolerant birdsfoot trefoil plants were also tolerant in vitro, (Swanson and Tomes 1980) suggested that a cellular tolerance mechanism existed and that it should be possible to select callus cultures with a similar mechanism of tolerance.

The objectives of this research were: (1) to determine if in vivo and in vitro responses to 2,4-D in red clover (*T. pratense* L.) were correlated, and (2) to obtain 2,4-D tolerant callus lines.

Materials and methods

Correlated response to 2,4-D

'Kenstar', 'Nolins', and six red clover half-sib families from the third cycle of a recurrent selection program for 2,4-D tolerance (Taylor et al. 1989) were utilized in a greenhouse and a tissue culture experiment to compare 2,4-D tolerance of each entry. The six half-sib lines had shown varying levels of whole plant 2,4-D tolerance in a previous test. In the greenhouse test, seeds of each entry were germinated in petri dishes and transplanted into Super-Cell Cone-tainers[1], each holding 150 cm^3 of sandy topsoil. The experimental design was a four-replicate, split-plot with 2,4-D concentrations as main plots and clover populations as subplots (four plants/subplot). Four weeks after transplanting, seedlings were inverted and immersed for 3 s in 500 ml of the respective 2,4-D solutions (0, 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 m*M*), which also contained 9.5% (v/v) ethyl alcohol and 0.1% (v/v) X-77 surfactant (alkyarylpolyoxyethylene glycols, free fatty acids, and isopropanol). Prior to treatment application, extra plots of each entry were harvested to establish a pretreatment dry weight (DW). Four weeks after treatment, above-ground plant parts were harvested, dried at 50 °C for 48 h, and weighed. Percent inhibition of each treatment was then calculated as [1-({final treatment DW – pretreatment DW}/{final control DW – pretreatment DW})]*100. This method was used so that only growth (weight change) over the treatment period was considered. Considering only weight change enhanced the accuracy of appraising the treatment effects.

The design of the tissue culture experiment was also a four-replicate split-plot with main plots as 2,4-D concentrations (0, 0.001, 0.01, 0.05, 0.1, 0.5, and 1.0 m*M*) added to the growth medium prior to autoclaving. Subplots were the red clover populations described previously (four hypocotyls per subplot). To obtain hypocotyls, seeds of the various entries were surface-sterilized and plated into 15 × 100 mm sterile petri dishes containing 25 ml of SGL nutrient medium (Phillips and Collins 1979). After germination and expansion of the cotyledons, 10-mm hypocotyl sections were removed from the seedlings and plated into 20 × 100 mm petri dishes containing one of the 2,4-D concentrations in 50 ml of L2 nutrient medium solidified with 8 g l^{-1} agar (Phillips and Collins 1979). Picloram (0.06 mg l^{-1}) and 6-benzyl aminopurine (0.01 mg l^{-1}) were included as regular components of the nutrient medium. Petri dishes were placed on shelves at 25 °C under low-intensity (50 μmol photon m^{-2} s^{-1}) fluorescent plant lights (16 h light/d). Six weeks later, the calli was dried, weighed, and data were converted to percent inhibition.

Data were analyzed by analysis of variance to test for population, 2,4-D concentration, and interaction effects. Since the interaction was significant, regression analysis was performed on percent inhibition and log-converted rates for each separate population. Best-fitting linear or quadratic relationships were chosen according to *F*-values, R^2 values, and significance of parameter estimates of the equations. Regression equations were developed to calculate I_{50} (predicted rate of 2,4-D required to cause 50% inhibition of growth) values for each line in each test. The predictive I_{50} values were then used as a relative measure of degree of tolerance – the higher the I_{50} value, the higher the level of tolerance. I_{50} values were ranked from highest to lowest in each experiment and a rank correlation was performed (Snedecor and Cochran 1967). Actual I_{50} values were also compared using simple correlation procedures.

Selection of 2,4-D tolerant cell lines

Callus derived from a single red clover plant selected from 'Arlington' (R578-OPC; supplied by G. B. Collins, University of Kentucky) was used as the source of callus in the selection experiments. Callus cultures of R578-OPC were utilized as explant inoculum to establish suspension cultures (Phillips and Collins 1980). These suspensions were grown in 125-ml glass flasks that were continuously rotated at 120 rpm at 25 °C and subcultured on a weekly basis. For the selection experiments, 5 ml of the suspensions were pipetted into 20 × 100 mm disposable petri dishes containing 50 ml of L2 medium supplemented with 0.18 m*M* 2,4-D. Preliminary experiments established that this rate caused 95% growth inhibition (data not shown). After 2–3 months, cells that had formed small calli on the selection medium were transferred to standard L2 medium for 2–3 months (cycle one tolerant lines). Then, small explants from the cycle one tolerant callus lines were rechallenged on L2 medium containing 0.18 m*M* 2,4-D. Sections of the callus tissue showing enhanced growth were selected and transferred onto standard L2 medium and designated cycle two tolerant lines. These were routinely subcultured every 4 weeks until ready for use in the evaluation experiments.

[1] Mention of a trade name is only for the purpose of identification and does not constitute a recommendation to the exclusion of other suitable substitute products.

Evaluation of 2,4-D tolerant cell lines

A 2,4-D bioassay procedure modified from standard auxin bioassay techniques described by Yopp et al. (1986) was developed to quantify levels of 2,4-D in selected and control callus lines. Hull-less oat (*Avena sativa* L.) seed (supplied by R. D. Barnett, North Florida Research and Education Center, University of Florida, Quincy) were used in all experiments for the source of coleoptile and internode tissue. Coleoptile tissue was obtained by germinating seed in the dark, supplemented with 2 h red light/day (25 W red light bulb) to suppress the growth of the first internode. Internode tissue was obtained in a similar manner but seed was not subjected to the red light treatment. After 72 h at 22°–25 °C, both sets of seedlings were ready for experimentation.

Coleoptile sections, 6 mm in length, were uniformly obtained using a tissue segment cutter (Mitchell and Livingston 1968). The sections were cut 3 mm below the tip of seedlings that were approximately 25 mm in length. The internodes were obtained in a similar manner but sections were cut starting at 2 mm below the coleoptile node. Coleoptile sections were rinsed for 1 h in distilled water containing 1 mg l^{-1} $MnSo_4 \cdot H_2O$. Internode sections were rinsed for 1 h in distilled water.

For all experiments, a basal aqueous medium of 20 g l^{-1} sucrose, 0.68 g l^{-1} K_2HPO_4, 1 g l^{-1} citric acid, and 8 g l^{-1} agar (pH = 5.0) was used for physical support of oat tissues and buffering. Twenty-five milliliters (3–4 mm of depth) of this solution was poured into disposable 20 × 100 mm petri dishes. The coleoptile and internode sections were then placed apical end up in the agar-solidified medium, with approximately 2 mm of tissue exposed to support either agar blocks or callus tissue.

To derive a response curve of oat tissue elongation to known concentrations of 2,4-D, agar blocks 15 mm^3 in size were cut from distilled water solidified with 20 g l^{-1} agar and placed on the apical end of either coleoptile or internode sections supported in agar as above. The blocks were injected with 1 µl of the following 2,4-D solutions: 0, 4.5×10^{-3}, 4.5×10^{-2}, 0.45, 2.25, and 4.5 m*M* using 0.95% (v/v) ethyl alcohol. The experimental design was a randomized complete block of three replications and was conducted twice. Coleoptile and internode sections were treated as separate experiments. Lids were placed on the dishes to prevent desiccation and the dishes were placed in the dark at 22 °C. After 24 h, elongation of the sections was measured using a caliper-type micrometer. The analysis of variance model tested experiments, 2,4-D concentration, and their interaction. Regression analysis was then used to develop equations to predict the concentration of 2,4-D that would cause a certain increase in length of the oat sections. Although the log of 2,4-D rates was also used in regression analysis, the actual rates provided the best models.

Red clover callus tissue selected as described previously was challenged for 6 weeks on L2 nutrient medium supplemented with 0.18 m*M* 2,4-D. Callus of 20 lines were divided into 0.25 g segments and placed on the medium in each of four replications. These tissues were then transferred to nutrient medium supplemented with 4.5×10^{-2} m*M* 2,4-D and after 4 weeks on this medium, the tissues were evaluated for 2,4-D activity using the bioassay technique described above.

Two experiments were conducted to evaluate selected callus-tissue response to 2,4-D. The first experiment used fresh callus tissue and the second used tissue which had been frozen and then thawed. Segments of callus approximately 3 mm in diameter (14 mm^3 volume) were placed directly on top of the vertically oriented coleoptile or internode sections in the agar plates. The experimental design was a four-replicate randomized complete block, with coleoptiles and internodes treated as separate experiments. Data from the experiments to evaluate selected callus tissue response to 2,4-D were analyzed by analysis of variance, and LSD ($P = 0.05$) was used to compare means of treatments to control means.

Results and discussion

Correlated response to 2,4-D

The lines chosen for this correlation study ranged in 2,4-D tolerance levels from very susceptible as whole plants (e.g., Nolins, Kenstar) to others which had expressed increased levels of tolerance (Table 1). For these lines, there was a significant rank correlation ($r_s = 0.79$, $P = 0.05$) and simple correlation ($r = 0.77$, $P = 0.02$) between whole plant and in vitro I_{50} responses to 2,4-D.

These experiments demonstrated that callus of red clover entries varied in response to the elevated 2,4-D levels. It was necessary to establish this fact so that appropriate selection pressure could be applied in order to select only 1%–5% of the population. The tolerance seen at the whole plant level was probably based on either increased metabolism or alteration at the site of action. If the tolerance were based on differential uptake, translocation, or intracellular sequestering, it more than likely would not translate to the cellular level. 2,4-D often stimulates ethylene production, which subsequently causes the initial responses (e.g., epinasty) to the herbicide (Ashton and Crafts 1981). Since the initial responses of whole plants in our previous work (Taylor et al. 1989) was visually similar regardless of eventual different levels of tolerance, this suggests that ethylene biosynthesis as a site of 2,4-D action was not changed. Thus, enhanced metabolism is strongly indicated as the basis of tolerance in plants which were able to recover more rapidly and outgrow the apparent damage.

These data provided sufficient initial justification to evaluate in vitro selection techniques to screen red clover tissue cultures for 2,4-D tolerance. If a genetically based system of 2,4-D metabolism can be selected in vitro, such as that demonstrated at the whole plant level, there is reason to believe that the tolerance would be present in regenerated plants.

Selection of 2,4-D tolerant cell lines

After 2–3 months, cellular aggregates (calli) could be identified that were capable of survival and growth on the 0.18 m*M* 2,4-D medium. These calli varied in size and appearance. To avoid selection of non-tolerant lines, it was necessary to subject these calli derived from these aggregates to a second cycle of selection. These procedures resulted in the isolation of 16-cycle two callus lines. Subclones of several lines were not carried through the second cycle of selection in order to compare the effectiveness of one cycle of selection versus two cycles.

Table 1. Whole plant and in vitro responses to 2,4-D of eight red clover genotypes

Half-sib family or cultivar	In vivo				In vitro			
	Regression			Rank	Regression			Rank
	I_{50}[a]	SE	R^2		I_{50}	SE	R^2	
	m*M*				m*M*			
L13,10,3	0.53	0.02	0.76[b]	1	0.07	0.02	0.64	2
L9,4,6	0.33	0.03	0.76	2	0.045	0.005	0.74	3
SL9,1,1	0.31	0.03	0.69	3	0.077	0.025	0.57	1
SR8,3,4	0.24	0.01	0.73	4	0.032	0.002	0.87	5
Nolins	0.23	0.02	0.89	5	0.03	0.002	0.89	6
Kenstar	0.21	0.03	0.78	6	0.018	0.001	0.89	7
L8,4s,1	0.18	0.01	0.81	7	0.039	0.003	0.97	4
R30,7,7	0.15	0.01	0.69	8	0.015	0.001	0.45	8

[a] I_{50} values are predicted values determined by linear or quadratic regression equations and represent rate of 2,4-D at which 50% growth inhibition occurs
[b] All regression models were significant ($P<0.001$)

Table 2. Internode and coleoptile responses to auxin-like activity associated with in vitro-selected red clover callus lines

Callus line[a]	Internode (mm)	Coleoptile (mm)
C0-01[b]	10.8	10.7
C1-05[c]	10.2	10.2
C1-08	9.9	9.9
C2-11	9.9	10.4
C2-13	9.6	9.7
C2-10	9.5	9.9
C2-01[d]	9.4	9.9
C2-16	9.4	10.5
C2-05	9.4	9.9
C2-08	9.3	9.9
C2-19	9.3	10.4
C2-07	9.1	10.3
C2-12	9.1	9.6
C2-14	9.1	9.3
C2-06	9.0	9.8
C2-03	8.9	10.1
C2-04	8.8	9.6
C2-15	8.5	9.5
C2-02	7.9	9.5
R578-OPC[e]	7.5	8.3
Control[f]	7.0	7.7
LSD (0.05)	1.0	0.7

[a] Callus lines are arranged in descending order according to internode lengths (i.e., callus lines causing the greatest elongation down to lines causing the least elongation)
[b] Callus tissue that had not been through any cycles of selection for 2,4-D tolerance, but was challenged on 2,4-D media for the bioassay evaluation tests
[c] C1 prefix indicates callus which had been through one cycle of selection for 2,4-D tolerance
[d] C2 prefix indicates callus which had been through two cycles of selection for 2,4-D tolerance
[e] Callus tissue that had never been on media supplemented with 2,4-D
[f] No callus tissue placed on bioassay tissue

The selection systems imposed on these cultures apparently altered characteristics other than the response to 2,4-D. In comparison to the control tissue, selected callus was less friable, darker in color and the growth rate of the cultures was reduced, regardless of the presence of 2,4-D in the medium.

Evaluation of 2,4-D tolerant cell lines

Three mechanisms of 2,4-D tolerance are possible at the callus level: (1) differential uptake and/or sequestering; (2) an altered site of action; and (3) metabolism. The use of a bioassay that determines the amount of 2,4-D present in the callus tissue as a measure of relative tolerance is based on the assumption that the tolerance mechanism is caused by differential metabolism. This assumes that the mode of action of 2,4-D is physiologically complex (Loos 1975). Enhancing tolerance to 2,4-D based on changes at the site of action would be improbable due to the complexity of this system. A similar argument could be made for a tolerance mechanism based on differential uptake and/or translocation. Both the aforementioned arguments and the literature (Jensen 1982) indicate that when an increase in 2,4-D tolerance has been observed, especially at the cellular level, the mechanism has been metabolic. Such a change could result from an alteration at a single gene, since only one enzyme is required to potentially metabolize a herbicide.

The 2,4-D bioassay procedure was well-suited to evaluate the response of callus tissue to 2,4-D. Control internodes and coleoptiles (those with no callus) did not show appreciable increases in length. The R578-OPC callus grown on standard L2 media (never exposed to 2,4-D) caused coleoptile growth which was 0.5–0.6 mm longer than the controls (Table 2). This may be attributed

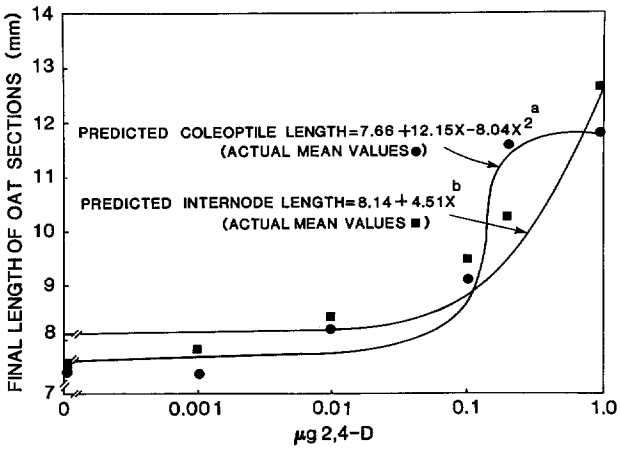

Fig. 1. Response of oat coleoptile and internode sections to known concentrations of 2,4-D. [a] $P > F = 0.0037$ and $R^2 = 0.98$. [b] $P > F = 0.0023$ and $R^2 = 0.92$

to the picloram in the L2 nutrient media which displays auxin-like properties. Callus line C0-01, which was not selected for tolerance but challenged on 2,4-D media for the evaluation tests, produced the greatest amount of elongation in three of the four experiments. This would indicate that this tissue has the greatest amount of susceptibility to 2,4-D. It is possible that C0-01 could not metabolize the 2,4-D that it took up, and consequently there were large amounts of 2,4-D present within the tissue. The average length achieved by the internodes (10.8 mm) and coleoptiles (10.7 mm) corresponds to 0.30 μg and 0.37 μg of 2,4-D, respectively, according to the standard response curve shown in Fig. 1.

Callus tissue that was subjected to only one cycle of selection (C1-05 and C1-08) caused internode elongation only slightly less than C0-01. The extent of coleoptile elongation induced by these two callus clones was intermediate between C0-01 and some of the least susceptible cycle two clones. The cycle two selections showed variable responses to 2,4-D. One of the more tolerant lines, C2-14, had an average length over both bioassay tissues of 9.2 mm. This corresponds to 0.18 μg of 2,4-D or a 61% decrease in 2,4-D concentration compared to the C0-01 (0.46 μg of 2,4-D) control. In the internode test, C2-15 had an average length of 8.5 mm, corresponding to 0.08 μg of 2,4-D. This would indicate that this line contains 83% less 2,4-D than the C0-01 control. These results demonstrate the importance of two cycles of selection.

The results of the first coleoptile test were different ($P < 0.05$) from the second. This is likely due to experimental procedure, in that there may have been differences related to using different callus tissue types (fresh versus frozen) in the two tests. The freezing served to disrupt membrane integrity as a means of preventing differential release of 2,4-D that may have occurred in fresh tissue. Thus, the freezing and thawing process could have provided a more accurate assessment of 2,4-D content. In contrast, results of the two internode tests were correlated ($r = 0.64$, $P = 0.001$). The rank correlation between the overall placement of the callus lines in the internode and coleoptile tests was also high ($r = 0.62$, $P = 0.01$).

Previous research with red clover (Taylor et al. 1989) demonstrated the usefulness of recurrent selection at the whole plant level to increase levels of 2,4-D tolerance. The research reported herein was undertaken to evaluate the utility of in vitro methods to select for herbicide tolerance in red clover. These data demonstrated that callus showing an increased tolerance to 2,4-D can be obtained via in vitro methods, but we were unable to regenerate whole plants from these 2,4-D tolerant calli.

In vitro selection has been regarded as a tool that plant breeders can use to reduce the amount of time spent screening plants in the field. This may be a particularly useful technique for use with inbred lines and if one is interested in locating a single gene trait. Thousands of plants may have to be screened by conventional methods before isolating a gene. In vitro selection could be more effective and efficient than whole plant screening in this situation. A second situation which might favor in vitro selection is one where whole plant variability has been limited and new sources of variability are needed. However, a prerequisite is the capability to regenerate whole plants from in vitro-selected germplasm. A current focus of our research is to develop a red clover population with higher regeneration potential.

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