The *in vitro* genetics of barley (*Hordeum vulgare* L.): Genetical analysis of immature embryo response to 2,4-dichlorophenoxyacetic acid

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The genetics of *in vitro* response of immature barley embryos was examined by means of diallel analysis. Both mean expression and sensitivity to 2,4-dichlorophenoxyacetic acid (2,4-D) were shown to be under genetic control. Additive and non-additive factors were involved in the control of *in vitro* response. The relative importance of these parameters was shown to change with increasing concentrations of 2,4-D. Response *in vitro* to media containing 2, or more, mg l⁻¹ of 2,4-D was characterised by large reciprocal differences. The implications of these findings for the manipulation of barley genotypes in culture are discussed.

INTRODUCTION

The application of tissue culture technology to the improvement of the barley crop depends upon the ability to initiate, maintain and manipulate *in vitro* cultures. Performance in culture of a range of crop plants has been shown to be influenced by cultural practices and media components (Bayliss and Dunn, 1979; Green, Phillips and Kleese, 1974; Sears and Deckard, 1982). More recently it has been confirmed that the genotype of an organism can influence its *in vitro* development. The ability to regenerate plants from callus culture has been shown to be under genetic control in such species as: alfalfa (Bingham *et al.*, 1975), red clover (Keyes, Collins and Taylor, 1980), wheat (Lazar, Collins and Vian, 1983), maize (Tomes and Smith, 1985) and barley (Hanzel *et al.*, 1985; Goldstein and Kronstad, 1986).

In a previous paper (Powell and Dunwell, 1987) factorial experiments were employed to assess the relative importance of genotype and media on the performance of barley immature embryos in culture. Such an approach may be used to identify barley genotypes amenable to culture. A further advantage to such experimentation is

that a genotype's sensitivity to culture conditions, in their broader context, may be ascertained. Powell and Dunwell (1987) have shown that a two-dimensional assessment in terms of mean performance and sensitivity is required in order to describe a genotype's performance in culture. The experiments reported in the present paper were designed to describe the extent and nature of the genetic control in mean response of immature barley embryos in culture and their sensitivity to varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D).

MATERIALS AND METHODS

Experimental material

A 5 × 5 diallel set of crosses (including parents and reciprocals) was performed between the following spring barley genotypes: 4082, Apex, Doublet, Heriot and Golden Promise. The material was maintained in a glasshouse with a minimum 16-hour photoperiod at a temperature of 20–30°C. Florets were emasculated by clipping the lemma and palea, the anthers were then removed using fine forceps (Pope, 1944). In order to avoid effects associated with clipping the lemma and palea during hybridisation, the parental genotypes were also clipped at anthesis.

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Culture of immature zygotic embryos

Caryopses containing embryos 1–2 mm in length were removed from the spike, sterilised for 15 mins in 2 per cent sodium hypochlorite, with Tween 20 to act as a wetting agent, and were then washed in sterile distilled water. Embryos were excised and placed with the scutellum uppermost on 20 ml of solidified medium (agar 0.7 per cent) at a frequency of five per petri dish (90 × 15 mm). The medium employed was based on that of Murashige and Skoog (1962) with the major salts at half the recommended concentration, 3 per cent sucrose and 2,4-D at 1, 2, 4 and 8 mg l⁻¹. Dishes were sealed with parafilm and incubated at 20 ± 2°C with a 16-hour photoperiod provided by white fluorescent tubes (30 µE m⁻² s⁻¹).

After 28 days in culture, each embryo and attached callus was harvested and its shoot and root length (SL and RL respectively) recorded. After blotting with paper tissues the fresh weight (F.Wt.) was then recorded. After drying overnight at 35°C, the dry weight (D.Wt.) was recorded and subsequently the water content (Wat) calculated.

Experimental design and statistical analysis

The experiment was a randomised complete block design with two replicates. Each genotype was represented by ten embryos within each environment (differing concentrations of 2,4-D). The statistical analyses were performed according to the methods described by Hayman (1954). The variance (*Vr*) and covariance (*Wr*) analysis developed by Jinks (1954) was also used in the genetic assessment of the variation. It should be noted that F.Wt., D.Wt. and Wat are not completely independent of one another in that the last is obtained by estimating the difference in the other two.

RESULTS AND DISCUSSION

Preliminary analyses of the data revealed large, significant correlations between mean and variance

(scalar effects) for all the characters measured indicating that the scales on which the characters were measured were inadequate. The data were therefore subjected to a square root and eventually fourth root transformation. The results of these transformations on the correlations between means and variances for the five characters are given in table 1. The $x^{1/4}$ transformation reduced the magnitude of the correlation to insignificance and subsequent analyses were based on the data transformed in this manner.

Mean performance for each character, averaged over the four concentrations of 2,4-D, has been examined by the analysis of variance of diallel tables. Genotypic sensitivity to the synthetic auxin 2,4-D was assessed by measuring a genotype's phenotypic variance over concentrations of 2,4-D and using the square root of the variance component (σ) as the metric for analysis. In this way it is possible to establish whether genotypes respond in the same way to increasing concentrations of 2,4-D. The results from the Hayman (1954) analysis of variance for both mean performance and sensitivity are given in table 2. Five main effects were examined (Mather and Jinks, 1982):

- a*—additive genetic variation
- b*—dominance variation
 - b*₁—mean dominance deviation, i.e., the overall difference between the *F*₁'s compared to their mid-parent values.
 - b*₂—the variation in mean dominance deviations of the *F*₁'s from their mid-parent values within each array, over arrays.
 - b*₃—dominance deviations that are unique to individual *F*₁'s.
- c*—average reciprocal differences.
- d*—further reciprocal differences not accounted for by *c*.

Considering mean performance, significant additive genetic variation was observed for F.Wt. and SL. Significant levels of dominance variation were detected for all the characters scored. The *b*₁ term is significant for F.Wt. while the *b*₂ and *b*₃ terms are significant for all the characters scored. However, the most striking feature of these analy-

Table 1 Correlation coefficients between mean and variance for the untransformed and transformed data

	F.Wt.	D.Wt.	Wat	SL	RL
Untransformed	0.80	0.82	0.71	0.66	0.81
Transformed $x^{1/2}$	0.53	0.38	0.47	0.41	0.58
Transformed $x^{1/4}$	0.17	0.32	0.17	0.25	0.45

Table 2 Results from the Hayman analysis of variance for mean performance ($\bar{x}$) and sensitivity (σ)

Item	df	F.Wt.		D.Wt.		Wat		SL		RL	
		$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
<i>a</i>	4	37.5**	17.4	33.6	21.6	28.0	10.7	929.9*	2519.8*	603.3	2375.1*
<i>b</i>	10	97.9***	25.4	105.5***	28.6	91.5*	24.8	1366.4***	1968.0	2593.6***	821.9
$\left\{ \begin{array}{l} b_1 \\ b_2 \\ b_3 \end{array} \right.$	1	40.9*	1.6	4.9	0.4	28.0	14.9	1050.7	4167.9*	520.3	1125.3
	4	134.2***	29.0	116.4***	40.7*	96.4*	25.2	1317.1**	556.1	2767.1**	349.7
	5	80.3***	27.3	117.0***	24.5	100.3*	26.4	1469.0***	2657.5*	2869.5***	739.1
<i>c</i>	4	256.6***	13.8	256.8***	85.9***	280.3***	14.6	1240.3***	994.8	1118.6	2312.8*
<i>d</i>	6	24.9*	49.5*	14.7	20.5	101.0*	52.2*	2044.3***	1477.0	1112.3	2152.2*
Reps	1	10.8	2.7	69.0*	81.9*	23.1	1.1	122.0	275.4	108.6	1.7
Pooled ints	24	8.0	13.7	16.2	13.9	30.5	12.8	297.2	928.1	544.9	632.7

* $P = 0.05 - 0.01$, ** $P = 0.01 - 0.001$, *** $P = < 0.001$

ses was the presence of highly significant reciprocal effects (*c*). The *d* item was also significant for F.Wt. and Wat. The significance of *a* for SL and RL indicated additive genetic variation for sensitivity with respect to these characters. Significant *c* effects were detected for D.Wt. and RL. Furthermore the *d* item was significant for F.Wt., Wat and RL. The significance of the *c* and *d* items demonstrates that the sensitivity of F_1 embryos in culture depended on the direction that the cross was made, i.e., there were significant reciprocal differences. With the exception of SL the b_1 item was non-significant, indicating that there were no detectable differences between the overall mid-parent values and their progeny means. Since σ (the phenotypic variance over concentrations of 2,4-D) was the variate analysed, it would suggest that there was no difference in the stability of homozygotes and heterozygotes to variation in 2,4-D concentration. The mean squares for variation of the five parental lines and the 20 F_1 hybrids over the four concentrations of 2,4-D are given in table 3. Only for the character SL ($VR = 1.83$, $P = 0.01 - 0.001$) did the F_1 hybrids appear to be more stable than the true-breeding parental lines. For the other characters there was no evidence of greater parental instability than observed in the F_1 's. These results are in agreement with those of Jinks and Mather (1955), Paxman (1956) and

Broadhurst and Jinks (1966) in suggesting that heterozygosity *per se* does not confer greater stability.

To investigate further the underlying genetic control, the covariance-variance (Wr , Vr) relationship (Jinks, 1954) was examined for both mean performance, averaged over concentrations of 2,4-D, and sensitivity. The variance (Vr), along with the covariance between F_1 's and the non-recurrent parent (Wr) of each array, was calculated for replicates separately and the $Wr + Vr$, $Wr - Vr$ analysis applied (Mather and Jinks, 1982). This revealed that the necessary criteria (i.e., no significant $Wr - Vr$ differences and significant $Wr + Vr$ effects) for both mean performance and sensitivity were only fulfilled in the case of SL and even for this character, in the $Wr + Vr$ analysis the ratio between the mean square for arrays and that for replicates was just formally non-significant ($P = 0.2 - 0.1$). The results of these analyses of variance together with the joint regression analyses are given in table 4. These indicated that estimates of the regression coefficients from the two blocks were consistent and in agreement with a line of unit slope. The significant non-additive genetic variation detected by the Hayman analysis of variance for these characters can mainly, therefore, be attributed to dominance effects rather than to non-allelic interactions.

Table 3 Mean squares for within genotype variation, i.e., stability, for the homozygous parents and their heterozygous F_1 's

	Characters				
	F.Wt.	D.Wt.	Wat	SL	RL
Variation within parents (30 df)	98.8	155.8	50.6	3902.1	3460.5
Variation within F_1 's (240 df)	110.3	139.7	80.3	2132.7	2619.3

The W_r , V_r points have been plotted for SL (after averaging over replicates) in fig. 1 for mean performance and in fig. 2 for sensitivity. Parents with the most dominant alleles fall nearer to the origin than those with the greater number of recessives (Mather and Jinks, 1982). Thus parent 5 (Golden Promise) contains the greatest number of recessive alleles controlling mean SL and its sensitivity to increasing 2,4-D. Crosses involving Golden Promise also exhibited the greatest sensitivities and thus dominance would appear to have been for lower sensitivity. Estimates of the components of variation for the two parameters measuring SL are given in table 4. The existence of dominance effects detected in the graphical analysis were confirmed by the finding that $(H_1/D)^{1/2}$ was approximately equal to one for both variates. In the case of mean performance H_2 was smaller than H_1 due to unequal allele frequencies, confirmed by the mean frequency ($uv = 0.20$) being less than its theoretical maximum of 0.25, which holds when frequencies are equal at 0.50. The positive value of F for this character shows that the dominant alleles tend to be commoner than their recessive counterparts in this sample of

Figure 1 The W_r , V_r graph for mean expression of shoot length

Figure 2 The W_r , V_r graph for the sensitivity of shoot length.

genotypes and a narrow sense heritability estimate of 38 per cent was obtained. For SL sensitivity H_2 is larger than H_1 and a narrow sense heritability of 9.36 per cent was obtained.

The present results demonstrate that both mean performance and sensitivity of *in vitro* response are clearly under genetic control and amenable to genetic analysis in the same way as more conventional characters. It is also of interest, however, to examine genotypic responses in each environment *i.e.*, in each concentration of 2,4-D. The mean scores for: F.Wt., D.Wt., Wat, SL and RL in each of four concentrations of 2,4-D are given in table 5, along with estimates of the genetic variation calculated from the expected mean squares. The greatest variation between genotypes for the characters F.Wt., D.Wt. and Wat was observed when 2 or more mg l^{-1} of 2,4-D were included in the media. It can also be seen that for these characters 2 mg l^{-1} resulted in maximum mean expression. This is in contrast to the genotypic response when measured in terms of SL and RL where maximum mean performance was observed in media containing 1 mg l^{-1} of 2,4-D. Shoot and root development would therefore appear to be very sensitive to levels of 2,4-D in excess of 1 mg l^{-1} and its concentration can be manipulated to induce a desired level of phenotypic expression.

The genetic variation observed in each environment may be examined further by the analysis of variance of diallel tables. The Hayman analysis of

Table 4 $Wr + Vr$ and $Wr - Vr$ analysis (a), as well as (b) regression analysis of Wr on Vr , for mean performance and sensitivity of SL

(a)	Mean performance SL			Sensitivity SL		
	df	MS(10^{-4})	P	df	MS (10^{-4})	P
$Wr + Vr$						
Between lines	4	103.87	0.1-0.05	4	154.30	0.2-0.1
Within lines	5	20.70		5	74.31	
$Wr - Vr$						
Between lines	4	3.68	N.S.	4	3.95	N.S.
Within lines	5	4.80		5	5.04	
(b) Joint regression analysis of Wr on Vr						
Regression	1	117.84		1	251.96	
Heterogeneity	1	1.26		1	2.82	
Remainder	6	6.24		6	5.95	
$\hat{b} = 0.9894 \pm 0.2278 \quad 1.0691 \pm 0.1643$						
$D =$		913.9			460.93	
$H =$		1366.70			891.17	
$H =$		1099.67			1115.15	
$E =$		297.21			928.15	
$F =$		1061.18			162.07	
$uv =$		0.25			0.31	
$\sqrt{H_1/D} =$		1.22			1.39	
Heritability $^\dagger =$		37.94%			9.36%	

† Estimated as: $1/4D/(1/4D + 1/4H_1 - 1/4F + E) \times 100$.

variance for each character in the four concentrations of 2,4-D is given in table 6. Considering the character of F.Wt., the a item was significant in the four concentrations of 2,4-D indicating the presence of additive genetic variance. Furthermore the magnitude of the additive genetic variation increased as the concentration of 2,4-D was

increased. The b item was also significant for this character in the four concentrations revealing dominance variation. The b_1 item was significant when tested in media containing 1 and 2 mg l $^{-1}$ of 2,4-D, while the b_2 item was significant for this character in all four concentrations. Significant dominance effects associated with particular F_1 's

Table 5 Estimates of the percentage genetic variation and mean scores in the four concentrations of 2,4-D

	Concentrations of 2,4-D (mg l $^{-1}$)			
	1	2	4	8
F.Wt.				
% Genetic variation	1.06	82.68	79.36	44.97
Mean (mg)	499.60	593.10	542.70	526.50
D.Wt.				
% Genetic variation	49.17	57.08	42.71	66.19
Mean (mg)	291.20	430.70	353.50	367.60
Wat				
% Genetic variation	56.02	77.17	82.32	55.46
Mean (mg)	485.60	525.80	506.90	477.40
SL				
% Genetic variation	74.97	71.93	72.19	56.21
Mean (mm)	2.02	1.81	0.67	0.45
RL				
% Genetic variation	74.28	60.79	68.63	70.47
Mean (mm)	1.24	1.07	0.29	0.19

Table 6 Hayman analyses of variance for the characters F.Wt., Wat, SL and RL in the four concentrations of 2,4-D

Item	df	F.Wt. (10^4)				D.Wt. (10^4)				Wat (10^4)			
		1 mg	2 mg	4 mg	8 mg	1 mg	2 mg	4 mg	8 mg	1 mg	2 mg	4 mg	8 mg
a	4	163.2*	100.4**	313.4***	435.6***	133.3**	20.7	163.0	534.1***	144.7***	130.3**	148.1***	176.1*
b	10	116.1*	184.0***	222.4***	238.8**	69.6*	270.2***	152.7	278.2*	101.9**	90.5***	170.1***	160.2**
$\left\{ \begin{matrix} b_1 \\ b_2 \\ b_3 \end{matrix} \right\}$	1	296.7*	257.6***	44.1	0.7	180.2*	418.4*	39.3	351.5*	266.1***	118.0*	29.5	79.1
c	4	128.6*	283.8***	106.5*	244.9*	93.0*	319.6**	42.7	276.7*	100.6*	169.0***	112.7**	139.9*
d	5	70.0	89.5**	350.9***	281.7**	28.8	201.2*	263.5*	264.7**	70.3*	22.2	244.3***	192.8**
Reps	4	81.1	489.5***	398.1***	426.4***	50.1	545.2***	635.8***	467.3***	66.1	281.7***	271.9***	265.7***
Pooled int	6	55.8	74.7**	132.7**	181.8*	42.8	30.0	115.3	177.3*	62.1	88.3**	181.3***	95.3
	1	323.5*	74.7	10.8	296.8*	62.0	940.8***	223.1	410.4**	260.1***	62.1	2.7	103.5
	24	43.0	18.4	28.1	61.3	24.0	58.6	90.5	66.5	26.3	16.5	18.06	47.1

Item	df	SL				RL			
		1 mg	2 mg	4 mg	8 mg	1 mg	2 mg	4 mg	8 mg
a	4	5.06***	4.72***	3.64***	0.95**	1.95***	1.62**	0.72***	0.40***
b	10	3.40***	2.83***	0.90*	0.35	1.52***	0.66*	0.23*	0.11*
b_1	1	1.99*	3.31*	0.63	0.20	1.07*	1.09	0.19	0.00
b_2	4	2.05**	3.32***	0.75	0.28	0.61*	0.71	0.21	0.16
b_3	5	4.76***	2.43***	1.07*	0.48	2.35***	0.54	0.24	0.09
c	4	2.25*	3.34***	2.81***	1.12***	1.10**	2.60***	0.86***	0.42***
d	6	1.20*	0.44	1.15**	0.75*	0.32	0.31	0.30**	0.20**
Reps	1	0.04	2.24*	0.19	0.14	0.01	0.85	0.16	0.05
Pooled int	24	0.42	0.43	0.28	0.19	0.18	0.26	0.08	0.04

* $P = 0.05-0.01$, ** $P = 0.01-0.001$, *** $P < 0.001$.

(b_3) was observed in the media containing 2, 4 and 8 mg l^{-1} . Similarly, reciprocal differences (c) were detected in these three environments.

Additive genetic variation was observed for the character D.Wt. in media containing 1 and 8 mg l^{-1} of 2,4-D. There was evidence for dominance variation in media containing 1, 2 and 8 mg l^{-1} . Specific reciprocal differences (c) were observed in media containing 2 or more mg l^{-1} of 2,4-D. A significant d item was observed in media containing 8 mg l^{-1} . With respect to Wat there were significant additive genetic differences between the genotypes at the four concentrations of 2,4-D. There was also evidence for dominance variation, including overall dominance (b_1), dominance within specific arrays (b_2) and dominance unique to particular F_1 's (b_3). Large reciprocal differences were expressed when embryos were cultured in media containing high levels of 2,4-D.

For both SL and RL, additive and non-additive effects were detected. Dominance variation decreased as the concentration of the auxin increased. Significant reciprocal effects (c) were detected in both SL and RL in the four media tested. Although it should be noted that the importance of this genetic component increased as the concentration of 2,4-D was raised.

Estimates for the genetical parameters obtained from the Hayman analyses of variance revealed the presence of significant levels of additive and non-additive genetic effects. Furthermore the relative importance of these parameters changed not only between characters but also between environments. Differences in environments in this case created by the inclusion of the synthetic auxin 2,4-D. An example of this change in the relative importance of the genetical parameters with increasing 2,4-D is shown in graphical form in fig. 3 for the character Wat. When 1 mg l^{-1} of 2,4-D was included in the media the most important genetical parameter was a —the additive genetic component. However, when 2 or more mg were included the c component dominated the genetic variation available for manipulation. The d component assumed greater importance when 4, or more, mg l^{-1} were included. This pattern of change in genetical parameters characterised the expression of the characters measuring callus proliferation.

An example of the change in the expression of the 2,4-D for the differentiation characters is given by SL in fig. 4. Both the d and b components decreased when the concentration of 2,4-D increased. However, for this character the additive genetic component was the most important

Figure 3 The effects of the differing concentrations of 2,4-D on the expression of the genetical parameters controlling water content (Wat).

Figure 4 The effects of the differing concentrations of 2,4-D on the expression of the genetical parameters controlling shoot length (SL).

parameter in three out of the four concentrations. The expression of reciprocal effects (c) was at a maximum in media containing 2 mg l^{-1} and gradually declined with increasing 2,4-D. However, it should be noted that at 8 mg l^{-1} this genetical parameter had the largest mean square. The graphical presentation of the data for SL indicates the relative ranking of the genetical parameters. Such a ranking system may be of value during *in vitro* selection procedures. Thus, a strategy designed to select for SL in the presence of 2,4-D would need to maximise the relative magnitude of the additive genetic variation as well, perhaps, as mean expression. Reference to fig. 3 indicates that the maximum additive genetic variation was expressed in media containing 1 mg l^{-1} of 2,4-D, this environment also corresponded to the one which induced maximum shoot development.

CONCLUSIONS

The effects of genotype on barley callus growth and differentiation have been reported previously (Goldstein and Kronstad, 1986; Hanzel *et al.*, 1985). However, this is the first reported study of a detailed genetical investigation of *in vitro* response in barley. The identification and screening of useful genotypes for callus growth and differentiation is a pre-requisite for the application of tissue culture techniques to barley breeding programmes. In a previous paper (Powell and Dunwell, 1987) it was emphasised that two aspects of a genotype's performance in culture must be considered, *i.e.*, mean performance and sensitivity. In the present report both these parameters were analysed and their underlying genetic control detailed. Additive and non-additive factors were clearly implicated in the determination of *in-vitro* response to 2,4-D. Furthermore, the relative importance of these parameters was shown to change in response to different media. *In vitro* response in media containing 2 or more mg l^{-1} of 2,4-D was characterised by the presence of large c and d effects, *i.e.*, large reciprocal differences. Such differences may not necessarily be due to cytoplasmic factors alone. Maternal or mother tissue effects may also be involved (Jinks, 1964). However, a distinction between these two genetic systems is important since genuine cytoplasmic control would suggest that DNA located in the chloroplasts or mitochondria is responsible for aspects of barley development in culture. It would also, of course, show a matrilinear pattern of

inheritance which could readily be amenable to manipulation.

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