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REGULAR ARTICLE

Design and analysis of a trial to select for stress tolerance

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ABSTRACT

To study stress tolerance of a set of breeding lines, it is necessary to evaluate the lines under stressed conditions and control conditions without stress. Thus, the evaluation involves two factors, i.e. stress treatment (with levels 'control' and 'stressed') and genotypes. There are many valid experimental designs for factorial experiments, which involve randomization of stress treatment-by-genotype combinations. Conducting trials laid out according to such standard designs may be difficult, however, due to potential neighbour effects between plots receiving different stress treatments. This frequently leads plant breeders to assess stress treatments in completely separated trials. For example, one trial may be conducted for the control treatment and one trial for the stress treatment. This approach limits the type of inferences that are available. In this paper it will be shown that inferences based on between-trial information are not possible. Inference based on within-trial information is feasible, however, as will be illustrated using an example. The practical implication is that a genotype-specific stress tolerance index cannot be estimated with this approach, but that a relative comparison of the genotypes' stress tolerance is possible.

Key Words: *split-plot design; split-block design; between-trial information; within-trial information; contrast; estimability; confounding.*

INTRODUCTION

Tolerance to drought stress is an important trait in wheat breeding programs. It is therefore of interest to study the genetic effects in breeding populations as related to drought tolerance. We here consider a trial that was conducted to evaluate all 21 genotypes generated from a 6×6 half diallel cross including self-crosses under stress and under control conditions without stress. The stress condition was induced by the use of potassium iodide, which

induces a dehydration stress. There were two treatment factors of interest: (i) stress level (control vs. dehydration stress) and (ii) genotypes (6 parents and 15 cross combinations). Two specific objectives of these two trials were:
i) to study the mode of gene action, i.e., whether it is influenced by the stress-induced condition or not;
ii) to compare both stress levels (control vs. dehydration stress) in order to study whether the selection under stress-induced conditions is effective or not.

Wheat plants display a unique mobilization of stem reserves to grains under drought stress. This response can be mimicked by applying potassium iodide (KI) at anthesis (Blum 1998), which induces dehydration stress. Clearly, the artificial dehydration stress is not exactly the same as drought stress, but it was used here as a convenient proxy to simulate such conditions. When applying this treatment in a field trial, great care is needed to make sure that non-stress plots are not affected as well. This can be achieved, e.g., by leaving sufficient guard space between stressed and non-stressed plots. These technical challenges need to be considered when designing the trial. The most commonly used approach in this situation is a split-plot design with stress level as the main plot factor and genotypes as the subplot factor. This design has a smaller number of direct adjacencies of stressed and non-stressed plots than a design that randomizes stress level-by-genotype combinations, but such adjacencies are not entirely avoided even with split plots. An alternative approach, also often used in such a case, is to conduct two separate trials side by side, one with the stress treatment and one with the non-stress treatment. Such an approach, however, does not allow a direct comparison of stressed and non-stressed treatments because there is a confounding of the trial area effect with the stress treatment effect.

Despite the limitations of the design regarding assessment of stress effects, some relevant information can be extracted from two separate trials. In this paper, we critically evaluate the options for and limitations of valid inferences to be made from such trials. It will be demonstrated that inferences based on within-trial information can be safely made from a joint analysis of both trials and that inferences based on between-trial information are not available due to limitations of the design.

MATERIALS AND METHODS

DESCRIPTION OF THE TWO TRIALS

The experimental materials comprised six parental wheat varieties/lines, namely, Nacozari, LLR 22, LLR 20, CB 42, Parula and LLR 21 and their 15 direct crosses. The two trials were planted side by side in fall 2012 and harvested in spring 2013 in the field area of the Department of Plant Breeding and Genetics, PMAS Arid Agriculture University Rawalpindi, Pakistan. In each of the two trials, all the F1 hybrids along with their parents were planted in the field in a randomized complete block design with three replicates. Both trials were sown under rain-fed conditions. The first trial served as a control, whereas the second trial was treated with potassium iodide at the 50% anthesis stage to create chemical desiccation, thus simulating drought stress. Row length was 5 m, and distances between rows and plants were 30 cm and 15 cm, respectively. Two seeds per hole were sown with the help of a dibbler and later thinned to one seedling per hill after germination. Other cultural and agronomic practices were kept uniform, i.e., seed treatment, time of sowing, weeding, thinning, fertilization etc. for the whole trial. At maturity, ten guarded plants from each replicate were selected randomly for recording data for the traits. In this paper, we consider the response variable 'number of grains per ear' for illustration of analysis options.

In the dehydration-stress trial, potassium iodide is used to induce desiccation by decreasing chlorophyll content, stomatal conductance and rates of photosynthesis and transpiration of flag leaves of the treated plants. Potassium iodide desiccates the plants' green parts in 2-3 days if used with 0.5% active ingredients. A safe distance from untreated

plots is necessary to avoid neighbour effect on untreated plots. We are aware that a split-plot design (Federer and King 2007) with dehydration-stress level as the main-plot factor could have been used to minimize neighbour effects and guard space needed. Even with this design, however, great care would have been needed in applying the dehydration-stress treatment to the main plots, and safe distances need to be kept from the untreated main plots. Because of these challenges, it was decided to plant two separate trials. This approach means that we have two independent trials, and hence the stress and control treatments cannot be directly compared. This limits the inferences concerned with dehydration stress tolerance.

BASIC MODELLING FOR TWO SEPARATE TRIALS

The basic model for a randomized complete block trial can be written as

$$y_{ij} = \mu + b_j + g_i + e_{ij}, \quad (1)$$

where y_{ij} is the observed response of the *i*-th genotype in the *j*-th block, μ is the general intercept, b_j is the effect of the *j*-th block, g_i is the effect of the *i*-th genotype, and e_{ij} is the plot error effect corresponding to y_{ij}. There are two levels of stress, tested in two adjacent trials, which need to be jointly analysed. The joint model can be written by just adding a subscript to identify the stress level. An important point to be made here is that the two trials do not only differ in the stress level applied, but also in the area to which the trials were planted. These areas will have an environmental effect on the response that is independent of the stress level applied to them. To make this point explicit, we add two subscripts here, i.e., a subscript *t* for the trial area and a subscript *s* for the stress level:

$$y_{ijst} = \mu_{st} + b_{jt} + g_{is} + e_{ijst}. \quad (2)$$

In our case, stress level *s*=1 is applied to trial area *t*=1 only and stress level *s*=2 is applied to trial area *t*=2 only, so there is complete confounding of area and treatment effects. Note that the block effects carry only the subscript *t*, indicating that this is a purely environmental effect that is not assumed to interact with the stress treatment. This is in agreement with the usual assumption of block-treatment additivity (or unit-treatment additivity; Hinkelmann and Kempthorne 1994, p.187). Conversely, the genotype effect only carries the stress level subscript *s*, for the same reason: it is a treatment effect and as such may interact with the other treatment factor "stress level", but not with design (block) factors, which are indexed by trial area *t* and blocks *j* within trial areas *t*. The intercept carries both the subscript *t* for trial and the subscript *s* for treatment, which reflects the confounding of treatment and trial effects. For evaluating the merits of the design with two separate trials, it is useful to consider the expected value of the sample mean of the *i*-th genotype under the *s*-th stress condition. This may be defined as

$$E(\bar{y}_{i\bullet st}) = \phi_{ist} = \mu_{st} + \bar{b}_{\bullet t} + g_{is}, \quad (3)$$

where $\bar{b}_{\bullet t}$ is the arithmetic mean of the block effects in the *t*-th trial. It is important to re-iterate that the trial-specific intercept μ_{st} comprises both the environmental effects of the *t*-th trial location and the treatment main effect of the *s*-th stress condition imposed. To make this more explicit, we partition the intercept μ_{st} as

$$\mu_{st} = \alpha_s + \beta_t, \quad (4)$$

where α_s is a main effect for the *s*-th stress level and β_t is a main effect (intercept) for the *t*-th trial area. With two separate trials, the effects α_s and β_t are completely confounded, and this is the essence of the limitation of this design.

Any stress tolerance index for a genotype that would be computed from ϕ_{i11} and ϕ_{i22} would suffer from this confounding (Kumar et al. 2012). For example, the simple difference is equal to

$$\phi_{i22} - \phi_{i11} = (\alpha_2 - \alpha_1) + (\beta_2 - \beta_1) + (\bar{b}_{\bullet 2} - \bar{b}_{\bullet 1}) + g_{i2} - g_{i1}. \quad (5)$$

The genetic difference of interest is

$$\gamma_i = (\alpha_2 - \alpha_1) + g_{i2} - g_{i1} \ . \quad (6)$$

Unfortunately, this is completely confounded with the purely environmental difference

$$\varepsilon = (\beta_2 - \beta_1) + (\bar{b}_{\bullet 2} - \bar{b}_{\bullet 1}). \quad (7)$$

An alternative view on the same problem is to consider the number of replications for the stress treatment. Generally, the number of replications per treatment is equal to the number of experimental units to which levels of the treatment factor were randomly allocated. In the case at hand, those units are represented by the two areas on which the two separate trials are conducted. Thus, the number of replications per treatment is one. The relevant error term for comparisons among the treatments is the variance among trial areas, but that variance cannot be estimated due to the lack of replication and the resulting confounding of treatment effects α_s and trial area effects β_t.

A BRIEF DETOUR: ALTERNATIVE JOINT EXPERIMENTAL DESIGNS

If we run a single trial, then we can directly estimate the genetic difference γ_i in (6). This is because in this case there is only one trial intercept and one set of block effects, so the environmental difference ε in (7) vanishes. As alluded to above, for a joint trial, one would preferably use a split-plot design with stress level on the main plots and genotype on the subplots. The error term for treatment comparison would be the main-plot error mean square. A second solution to the problem would be a replication of the pairs of trial at multiple locations. In this case there would be multiple realizations of the trial main effect β_t per treatment, meaning that a variance for trial effects could be estimated, thus providing a valid error term for treatment comparisons.

INTERACTION CONTRASTS FOR TWO SEPARATE TRIALS

Let us recall and emphasize that an important assumption of the joint analysis by model (2) is that there is no interaction between trial and genotype. On a small area of land and when the two trials are planted next to each other, this is usually a reasonable assumption. Note that we would readily make that assumption if a single trial were planted on the same area of land, for example a split-plot design with treatments as main-plot factors as suggested in the preceding section. In fact, no valid statistical analysis would be forthcoming without that assumption (Hinkelmann and Kempthorne 1994).

The assumption of block-treatment additivity does provide a way forward to obviate the confounding of stress treatment and trial effect even when two independent trials are conducted side-by-side. In each trial we may consider genotype differences. For example, assume we want to compare the genotypes *i* and *h*. The difference in a trial *t* is given by

$$\phi_{ist} - \phi_{hst} = g_{is} - g_{hs}\,, \quad (8)$$

which is free of environmental effects, so this difference is said to be estimable. Note that if we plug in the expression for ϕ_{ist} from (3), the trial main effect as well as the block mean cancel, which shows that the contrast is estimable from within-trial information. In order to compute genotype-specific measures, it is convenient to look at the difference of a genotype to the mean of all genotypes for a given stress level *s*, which is also estimable from within-trial information. This contrast can be defined as

$$\phi_{ist} - \bar{\phi}_{\bullet st} = g_{is} - \bar{g}_{\bullet s}. \quad (9)$$

A relative measure of stress tolerance can now be defined by comparing these differences between the two stress levels $s = 2$ and $s = 1$ as follows:

$$ST_i = g_{i2} - \bar{g}_{\bullet 2} - (g_{i1} - \bar{g}_{\bullet 1}). \quad (10)$$

This index ST_i is estimable from two independent trials because it is an interaction contrast that corresponds to a difference of two differences, each of which is estimable within one of the trials. It is therefore also based entirely on within-trial information.

It should also be stressed, however, that ST_i is just a relative measure that assesses the stress tolerance of one genotype relative to the others. Thus, if we find $ST_i = 1$, we only know that the tolerance of genotype *i* is one unit above that of the average tolerance of all genotypes. What we cannot assess in absolute terms is the genetic difference γ_i, which would tell us how the mean yield changes for the *i*-th genotype between stress and non-stress condition. For assessing this contrast, we would need to run a single trial with both stress and non-stress treatments randomized together, e.g. as a split-plot trial as suggested earlier. In the current trial, γ_i in (6) is a between-trial contrast and as such is not estimable because of lack of replication and because of the confounding of treatment and trial area effects.

Subsequently, we consider how the two questions posed in the introduction can be answered. It will be illustrated that answers are forthcoming by assessing contrasts that are entirely based on within-trial information.

QUESTION 1: IS GENE ACTION INFLUENCED BY STRESS LEVEL?

To answer this question, we partition the genotypic effect g_{is}, which is nested within stress level *s*, into a main effect for genotype (g_i) and an interaction between genotype and stress level $(\alpha g)_{is}$, i.e.,

$$g_{is} = g_i + (\alpha g)_{is}. \tag{11}$$

If gene action is influenced by stress level, there should be an interaction $(\alpha g)_{is}$, but not otherwise. We can test hypotheses about the interactions $(\alpha g)_{is}$ because these do not depend on the main effect α_s for stress level *s*, which itself is not estimable.

QUESTION 2: IS INDIRECT SELECTION FOR PERFORMANCE UNDER TOLERANCE BETTER THAN DIRECT SELECTION?

Suppose that we want to select for performance under stress treatment *s* = 2, and we consider either direct selection under the stress treatment *s* = 2 or indirect selection under the control treatment *s* = 1. Then the ratio of correlated response to selection under control treatment *s* = 1 (CR) and response to direct selection under stress treatment *s* = 2 (DR) is given by (Atlin and Frey 1990, Atlin et al. 2000)

$$\frac{CR}{DR} = \rho_g \sqrt{\frac{H_1}{H_2}}, \tag{12}$$

where H_1 and H_2 are the broad-sense heritabilities for treatments *s* = 1 and *s* = 2, respectively, and ρ_g is the genetic correlation. The broad-sense heritability for the *s*-th stress level evaluated using an RCBD is defined as

$$H_s = \frac{\sigma^2_{g(s)}}{\sigma^2_{g(s)} + \sigma^2_{e(s)}/n}, \tag{13}$$

where $\sigma^2_{g(s)}$ is the *s*-th genetic variance, $\sigma^2_{e(s)}$ is the *s*-th error variance, and n is the number of replicates per trial. The genetic variances $\sigma^2_{g(s)}$ under the two stress levels and the correlation ρ_g between the two stress levels are estimable because they do not depend on the main effect α_s for stress levels.

ACCOUNTING FOR THE DIALLEL STRUCTURE

To account for the diallel structure, we can partition the genotype effect into general combining ability (GCA) and specific combining ability (SCA) effects (Piepho 2013). The linear model can be extended accordingly. Thus, there are main effects for GCA and SCA as well as interactions treatment-by-SCA and treatment-by-GCA. These can be modelled either as fixed (Question 1) or random (Question 2).

RESULTS

INDIVIDUAL TRIALS

There are significant genotype effects (Table 1), and the error variances are somewhat different between the two treatments although the difference is not significant (Table 2). Nevertheless, taking a conservative approach, all analyses were performed assuming heterogeneity of variance between stress and non-stress conditions. For illustration, we report genotype means for the two trials for total dry matter at anthesis in Table 3. There are also significant GCA and SCA effects (Table 1), so there is scope for studying these genetic effects in relation to questions 1 and 2.

Table 1. Analysis of variance (ANOVA) of individual trials for number of grains per ear (sequential sums of squares).

Trial / Source of variation	Degrees of freedom	F-value	P-value
Control			
Replicates	2	1.45	0.2464
Genotypes	20	39.29	<.0001
GCA	5	22.12	<.0001
SCA	15	45.01	<.0001
Drought stress			
Replicates	2	0.61	0.5480
Genotypes	20	22.75	<.0001
GCA	5	10.80	<.0001
SCA	15	26.73	<.0001

Table 2. Error variances of individual trials for number of grains per ear and LR test for homogeneity of variance.

Trial		Variance estimates / LR test result
Control		11.56
Drought stress		8.60
LR-test for homogeneity of variance	χ^2	0.87
	p	0.3511

JOINT ANALYSIS OF BOTH TRIALS: ANSWERING QUESTION 1 TAKING GENOTYPES AS FIXED

There is significant treatment × genotype interaction (Table 4), so gene action is influenced by stress level. There also is significant interaction for both the GCA and SCA effects (Table 4), further endorsing this conclusion.

To further study the interaction, we defined a dummy variable x, which we set to $x = 0$ for treatment 1 and to $x = 1$ for treatment 2. Thus, the effect "genotype*x" assesses the difference "treatment 2 minus treatment 1" of a genotype. If treatment 1 is the control, then this is the reduction due to stress. This effect itself is not estimable because of the confounding of treatment and trial main effects as explained above (see Material and

Methods section). We can estimate interaction contrasts, however, defined as pairwise differences among genotypes for the effects "genotype*x". We can also compare this effect against the mean over genotypes, which corresponds to the stress index ST_i (Table 5).

Table 3. Genotype means per treatment for number of grains per ear. Means in a column followed by a common letter are not significantly different by the Tukey test controlling the family-wise type I error rate at α=5%. HSD = Tukey's honestly significant difference.

Genotype	Control	Drought stress
1×1	47.7^{cd}	35.0^{abcd}
1×2	42.7^{cdefg}	27.0^{cdefg}
1×3	24.7^{ij}	26.7^{defg}
1×4	51.0^{bcd}	40.0^{a}
1×5	17.0^{j}	18.3^{gh}
1×6	35.0^{fghi}	18.0^{gh}
2×2	50.0^{bcd}	40.7^{a}
2×3	36.3^{efgh}	22.7^{fgh}
2×4	51.3^{bcd}	39.7^{ab}
2×5	59.7^{ab}	33.7^{abcde}
2×6	43.3^{cdefg}	24.7^{fg}
3×3	52.7^{abc}	30.3^{cdef}
3×4	48.7^{cd}	36.0^{abc}
3×5	41.3^{defgh}	30.7^{bcdef}
3×6	50.7^{bcd}	14.7^{h}
4×4	33.0^{ghi}	27.0^{cdefg}
4×5	17.7^{j}	19.0^{gh}
4×6	32.0^{hi}	25.3^{egf}
5×5	62.3^{a}	39.7^{ab}
5×6	46.3^{cde}	36.0^{abc}
6×6	44.3^{cdef}	35.3^{abcd}
HSD(5%)	10.60	9.14

Table 4. F-tests (sequential, Type I) of effects for replicate-treatment, genotype and genotype-treatment interaction in joint analysis of number of grains per ear for both trials.

Source	Degrees of freedom	F-value	P-value
Replicate × treatment	5	100.66	<.0001
Genotype	20	51.78	<.0001
GCA	5	22.96	<.0001
SCA	15	61.38	<.0001
Genotype × treatment	20	12.69	<.0001
GCA × treatment	5	11.62	<.0001
SCA × treatment	15	13.05	<.0001

For example, from this result it emerges that 2×5, 3×3, 3×6, and 5×5 are particularly stress intolerant relative to the mean tolerance of all genotypes. But we have no absolute assessment that can quantify the overall effect of stress level or the effect of stress level for each individual genotype. Clearly, the contrast estimates in Table 5 represent a purely relative assessment, which may not be fully satisfactory for a full quantification of stress tolerance in absolute terms.

Table 5. Estimates of stress-tolerance index ST_i, defined as difference in number of grains per ear between control and drought treatment for contrast "entry vs. all entries" (equation 10) with standard error (SE). The p-values are adjusted for multiplicity using the simulation-based Edwards-Berry method.

Genotype	Estimate	P-value
1x1	0.0667	1.0000
1x2	-3.0833	0.9948
1x3	15.4667	<.0001
1x4	1.8167	1.0000
1x5	14.7667	<.0001
1x6	-4.4833	0.8503
2x2	3.5667	0.9779
2x3	-0.9833	1.0000
2x4	1.1167	1.0000
2x5	-13.9333	<.0001
2x6	-6.2333	0.3404
3x3	-10.0833	0.0047
3x4	0.06667	1.0000
3x5	2.1667	1.0000
3x6	-24.4333	<.0001
4x4	7.0667	0.1712
4x5	14.7667	<.0001
4x6	6.3667	0.3065
5x5	-10.4333	0.0026
5x6	2.5167	0.9995
6x6	3.9167	0.9468
SE	2.6566	

Table 6: Variance parameter estimates, heritabilities and ratio of correlated response (CR) over direct response (DR) to selection for number of grains per ear.

Parameter / quantity	Estimate
$\sigma^2_{g(control)}$	147.55
$\sigma^2_{g(drought\ stress)}$	62.38
$\rho_{g(control),g(drought\ stress)}$	0.6846
$\sigma^2_{e(control)}$	11.56
$\sigma^2_{e(drought\ stress)}$	8.60
$H_{control}$	0.9745
$H_{drought\ stress}$	0.9560
CR/DR	0.6912
σ^2_{GCA}	334.24
σ^2_{SCA}	53.37
$\sigma^2_{GCA\times treatment(control)}$	381.91
$\sigma^2_{SCA\times treatment(control)}$	54.90
$\sigma^2_{GCA\times treatment(drought\ stress)}$	141.15
$\sigma^2_{SCA\times treatment(drought\ stress)}$	6.04

JOINT ANALYSIS OF BOTH TRIALS: ANSWERING QUESTION 2 TAKING GENOTYPES AS RANDOM

Table 6 shows the estimates of variance parameters and derived quantities. The target treatment here is the stress treatment 2, so we consider direct selection under treatment 2 compared to indirect selection under treatment 1. The result shows that direct selection under treatment 2 is clearly preferable (Table 6) because the genetic correlation with treatment 1 is too low to exploit the slightly higher heritability under treatment 1. The GCA-related variances are larger than the SCA-related variances, indicating that selection for GCA is worthwhile.

CONCLUSION

We have shown that the original research questions 1 and 2 could be answered with the two separate trials. This is for two reasons: because all relevant contrasts and effects can be estimated from within-trial information, and because each trial has a valid randomization layout and full replication. By contrast, a stress-tolerance index is not estimable because this would require dwelling on between-trial information. This information does not yield information about the treatment effects due to a lack of replication for treatments and an associated confounding of trial area effects and treatment effects. If such an index needs to be estimated, an alternative design would be needed that provides true replication for treatments. One option is a split-plot design that randomly allocates treatment levels to main plots. A variation to this would be a strip-plot or split-block design, in which one factor would be allocated to rows and the other to columns within complete replicates (Federer and King 2007). Alternatively, a replication of the pairs of trials at multiple locations could be considered. Whenever feasible, we would recommend preferably using one of these alternative designs.

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