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Statistical analysis and field evaluation of the type 2 modified augmented design (MAD) in phenotyping of flax (*Linum usitatissimum*) germplasms in multiple environments

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Abstract

The type 2 modified augmented design (MAD) was used to phenotype seed yield, oil content and fatty acid compositions in a collection of 120 flax genotypes at two locations during three years. All six experiments had the same design, in which whole plots were arranged in 10 rows and 10 columns and each whole plot was split into five paralleled rectangular subplots with a control subplot in the centre of each whole plot. Two additional subplot controls were allocated at random in each of five randomly selected whole plots. Relative efficiency (RE) of adjusted versus unadjusted observations was evaluated for all six experiments. The RE was redefined as a ratio of pooled variance within both plot and subplot controls of the unadjusted values to that of the adjusted values. Two adjustment methods based on the row and column effect of plot controls (M1) and the regression of the test plots on the plot control (M3), were assessed to adjust for soil heterogeneity. The analysis of variance (ANOVA) results revealed that either M1 or M3 alone failed to sufficiently eliminate effects due to both additive and non-additive soil variation across the field. A combined method (M1+M3) appeared to be the most effective in most cases. The redefined RE can be used as an indicator of adjustment efficiency. A joint analysis of 120 flax genotypes over four environments showed that seed yield was significantly affected by environments and had significant interaction of genotype × environment. High yield mean and low coefficients of variation over multiple environments compared with a control cultivar are indicators of a stable and high yielding genotype, whereas oil and linolenic acid content were relatively stable traits. The automated statistical analysis of MAD with the corrected ANOVA and improved observation adjustment was implemented with SAS software and Perl scripts, which are freely available at http://probes.pw.usda.gov/bioinformatics_tools/MADPipeline/index.html.

Keywords: modified augmented design; analysis of variance (ANOVA); phenotyping; seed yield; oil content; linolenic acid; flax.
Abbreviations: ANOVA- analysis of variance; MAD- modified augmented design; RCBD- randomized complete block design; CV- coefficient of variation; AOAC- Association of Official Analytical Chemists; OIL- seed oil content; LIN- linolenic acid content; DF- degree of freedom; MS- mean square; EMS- expected mean square; SK- Saskatoon in Saskatchewan, Canada; MD- Morden in Manitoba, Canada.

Introduction

Randomization, replication, and local control (or blocking) are three basic principles in field experimental design for being able to estimate experimental errors and treatment means without bias, which have resulted in the extensive usage of complete experimental designs in agricultural research such as the randomized complete block design (RCBD) and the Latin square design (Fisher, 1971). However, in the early stages of breeding selection, a large number of test lines and a small amount of seed supply limit the use of experimental designs with replications. Augmented designs are nonreplicated experimental designs that circumvent this problem (Federer, 1956; Federer et al., 1975; Federer and Raghavarao, 1975). To further improve the efficiency of this type of experimental designs, Lin and Poushinsky (1985) proposed a modified augmented design (MAD) for rectangular plots (type 2). The design is structured as a split-plot with whole plots arranged in rows and columns. Each whole plot is split into five parallel rectangular subplots with a centre subplot for a control cultivar, called the plot control. In addition, two additional cultivars serve as subplot controls in some randomly selected whole plots. Test lines are randomly allocated to the remaining subplots which are called test plots (Fig 1). The control plots are used to estimate the row (R) and column (C) effects as well as plot error (the R × C interaction effect) to test for additive soil variation in the row and/or column directions of the test plots. The subplot controls are used to estimate the subplot error and to test for an R × C interaction effect, i.e., non-additive soil variation in multiple directions of the field. Three methods were proposed to adjust the test lines to mitigate the effects due to soil heterogeneity (Lin and Poushinsky, 1983, 1985; Lin and Voldeng, 1989), among which, Method 1 based on the row and column effects of control plots, and Method 3 based on the regression of the test plots on the control plots, were suggested for the type 2 MAD (Lin et al., 1983; Lin and Poushinsky, 1985; Lin and Voldeng, 1989). The MAD has been applied to and evaluated in crops, such as wheat (Snijders, 2002; Golparvar and Ghasemi-Pirbalouti, 2008), potato (Schaalje et al., 1987), soybean (Lin and Voldeng, 1989), barley (May et al., 1989; May and Kozub, 1995), sugarcane (Milligan and McDonal, 1990; Bhagyalakshmi and Somarajan, 1999) and maize (Afolabi et al., 2007). However, some issues arose from these studies. First of all, the statistical procedure of the MAD is not straightforward and

no detailed description of statistical analysis for the type 2 MAD is available. Though two SAS programs for the analysis of augmented design were reported (Scott and Milliken, 1993; Wolfinger et al., 1997), an automated computer program for the MAD is necessary. Second, all of these applications were based on analyses of individual yield trials for screening of superior breeding lines. When the same set of test lines and control cultivars are evaluated across locations and years, a joint analysis over multi-environments will help to obtain information about yield stability of test lines. Furthermore, the efficiency of a MAD was measured by relative efficiency (RE), which was calculated as a ratio of variances between the unadjusted and adjusted values of the subplot controls (Lin and Poushinsky, 1985). Since the number of subplot controls is limited, the RE estimates lack in precision, thus impacting the consistency of the best adjustment method suggested by ANOVA and by RE (Lin and Voldeng, 1989; May et al., 1989). The objectives of the present study were to develop an automated computer program package for the statistical analysis of the type 2 MAD combining SAS software and Perl scripts, and to evaluate the use and efficiency of the type 2 MAD in identifying phenotypes of flax germplasm based on the datasets of multiple traits generated over multiple environments.

Results

ANOVA of individual experiments

The ANOVA (Table 1) for flax seed yield showed that soil heterogeneity existed in 3 out of the 4 experiments in two years and two locations except in the 2011/MD experiment. Soil heterogeneity in these experiments were additive and only in one direction (row) except for the 2010/SK experiment which showed soil variation in both row and column directions. No significant R × C interaction effects were observed in 3 out of the 4 experiments, indicating no non-additive soil variation for seed yield in most of the experiments. The only exception was found in the 2010/MD experiment. A similar response was observed for oil (OIL) and linolenic acid (LIN) content in six experiments carried out over three years and at two locations (Table 1).

Adjustment of observations

Since there was significant soil variation in the test plots, an adjustment method must be applied to properly adjust the observed values of the test genotypes. Two methods, Method 1 (M1) based on design structure of plot control, and Method 3 (M3) based on covariance adjustment (Lin and Poushinsky, 1985; Lin and Voldeng, 1989), were previously used to adjust for soil heterogeneity. To compare the adjustment efficiency of the two methods, we first used them separately to adjust the observed values of all the genotypes, including the test genotypes and the control cultivars. Then the same ANOVA was performed for the data adjusted by M1 and by M3, respectively. Finally, we compared the mean squares (MS) and their statistical significance for all sources of variation: row, column and R × C (plot error). The results indicated that, regardless of the significance of row and/or column effects, adjustment by M1 reduced the MS values of row and column to nearly zero for all traits, but failed to eliminate the variation due to the R × C interaction (Table 1). Similarly, adjustment by M3 significantly eliminated the variation due to the R × C interaction, and reduced the MS values from row and column to some extent, but row and/or column effects were still significant. Either M1 or M3 alone failed to sufficiently eliminate the effects of the additive and non-additive soil variations when both row and column variations and the R × C interaction were significantly large. Subsequently, we propose a method that combines M1 and M3 (M1+M3) where adjustment by M1 first is followed by M3, where the latter adjustment is based on the adjusted values from M1. The results revealed that M1+M3 was able to better eliminate soil variation from the row and column directions and the R × C interaction in 13 out of the 16 experiments even though their effects in the row and column and the R × C interaction were not significant (Table 1).

In previous studies, the efficiency of the MAD was indicated by the RE which was defined based on the subplot controls (Lin and Poushinsky, 1983, 1985; Lin and Voldeng, 1989). In this study, five whole plots were selected for subplot controls to estimate subplot error and the degree of freedom (DF) for subplot error was only 8 (Table 1 and 2). As an example, Table 2 showed unadjusted (observed) and adjusted values of seed yield (g m^{-2}) of subplot controls (the plot control was also treated as subplot control) and their ANOVA results in the 2010/SK experiment. The results indicated that adjustment by M1, M3 or M1+M3 obviously reduced differences among plots (soil heterogeneity) represented by differences between means of plots and MS between plots in each adjustment method, but did not change MS between controls and MS of subplot error (retaining the same values for all adjustment methods). Consistent results were also shown in Table 1. Although adjustments using different methods were obviously efficient (because of reduced MS between plots in Table 2 or reduced MS between rows and/or between columns in Table 1), the MS of subplot controls (error) remained unchanged, suggesting that there is no obvious correlation between adjustment efficiency and subplot error, and subplot controls cannot be used alone for RE estimation. Therefore we redefined the RE as a ratio of pooled variance within both plot and subplot controls of the unadjusted values to that of adjusted values. Since plot controls are systematically allocated in the entire field and the purpose of adjustment is to remove the effects due to soil variation among whole plots (the basic assumption is that soil variation within a whole plot is homogeneous), the change of variance within plot and subplot controls after adjustment should be able to measure the efficiency of an adjustment method. The results showed that the redefined RE was able to verify most of the results determined manually by ANOVA (Table 3).

An appropriate adjustment should result in a decrease in coefficients of variation (CV) within plot and subplot controls. The RE estimates in the experiments were completely consistent with the change of CV in the plot controls, but not in the subplot controls (Table 3), which confirmed that subplot controls alone were insufficient for the RE estimation in this study. Therefore, the redefined RE can be used as a criterion to select the best adjustment method in the automated data analysis computer program.

Joint ANOVA of multiple experiments

In the present study, six experiments in three consecutive years at two locations were conducted to evaluate a set of flax germplasm. A fixed model for all factors, year, location and genotype was used to conduct a joint ANOVA (see Materials and Methods). The joint ANOVA results for yield,

Table 1. ANOVA of unadjusted (Unadj.) and adjusted values by Method 1 (M1), Method 3 (M3) and a combined method (M1+M3) .

Experiment	Source	Yield (g m^{-2})					OIL (%)					LIN (%)				
		DF	MS				DF	MS				DF	MS			
			Unadj.	M1	M3	M1+M3		Unadj.	M1	M3	M1+M3		Unadj.	M1	M3	M1+M3
2009/MD	Row						9	1.40**	0.00	0.03**	0.00	9	6.16**	0.00	1.66**	0.00
	Column						9	0.34	0.00	0.01	0.00	9	1.19	0.00	0.32	0.00
	R × C						80	0.31*	0.31*	0.01	0.04	80	0.72	0.72	0.19	0.66
	Error						7	0.08	0.08	0.08	0.08	7	0.32	0.32	0.32	0.32
2009/SK	Row						9	0.22*	0.00	0.13*	0.00	9	0.55	0.00	0.26	0.00
	Column						9	0.12	0.00	0.07	0.00	9	0.43	0.00	0.20	0.00
	R × C						81	0.10	0.10	0.06	0.00	81	0.55	0.55	0.26	0.29
	Error						8	0.05	0.05	0.05	0.05	8	0.33	0.33	0.33	0.33
2010/MD	Row	9	27.02**	0.00	2.24**	0.00	9	6.99**	0.00	5.26**	0.00	9	24.99**	0.00	2.36**	0.00
	Column	9	5.68	0.00	0.47	0.00	9	1.12	0.00	0.84	0.00	9	0.74	0.00	0.07	0.00
	R × C	81	4.67*	4.67*	0.39	1.07	81	0.84	0.84	0.63	0.96	81	0.71	0.71	0.07	1.42
	Error	6	1.11	1.11	1.11	1.11	6	0.26	0.26	0.26	0.26	6	0.45	0.45	0.45	0.45
2010/SK	Row	9	11.35**	0.00	9.86**	0.00	9	2.86**	0.00	0.08**	0.00	9	0.46	0.00	0.02	0.00
	Column	9	27.77**	0.00	24.13**	0.00	9	0.37	0.00	0.01	0.00	9	0.18	0.00	0.01	0.00
	R × C	80	4.06	4.06	3.53	2.83	79	0.61*	0.61*	0.02	0.00	79	0.38	0.38	0.02	0.19
	Error	6	2.62	2.62	2.62	2.62	5	0.08	0.08	0.08	0.08	5	0.12	0.12	0.12	0.12
2011/MD	Row	9	4.12	0.00	2.62	0.00	9	0.30	0.00	0.11	0.00	9	1.04*	0.00	2.11*	0.00
	Column	9	4.00	0.00	2.54	0.00	9	0.22	0.00	0.08	0.00	9	0.57	0.00	1.16	0.00
	R × C	81	5.55	5.55	3.53	3.78	81	0.35	0.35	0.13	0.12	81	0.51	0.51	1.04	0.54
	Error	8	2.85	2.85	2.85	2.85	8	0.25	0.25	0.25	0.25	8	0.34	0.34	0.34	0.34
2011/SK	Row	9	20.71**	0.00	1.28**	0.00	9	0.11	0.00	0.01	0.00	9	0.48	0.00	0.95	0.00
	Column	9	2.88	0.00	0.18	0.00	9	0.41**	0.00	0.05**	0.00	9	0.83**	0.00	1.65**	0.00
	R × C	81	3.17	3.17	0.20	0.06	81	0.12	0.12	0.01	0.00	81	0.28	0.28	0.56	0.38
	Error	8	1.12	1.12	1.12	1.12	8	0.07	0.07	0.07	0.07	8	0.31	0.31	0.31	0.31

* and ** represent significance at the 5% and 1% probability level. MS: mean square; DF: degree of freedom. The DF of error in some experiments is less than 8 because of missing values.

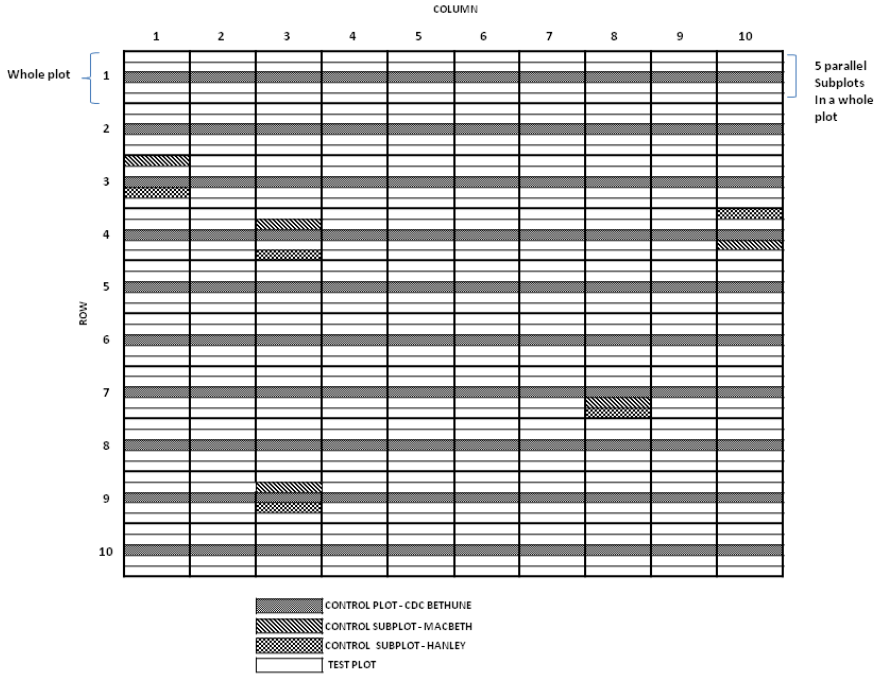

Fig 1. A diagram of field layout of the type 2 MAD for flax experiments. Plots were arranged in 10 × 10 grids. Each plot was split into five subplots with a plot control cultivar 'CDC Bethune' in the centre subplot. Two additional subplot controls, 'Macbeth' and 'Hanley' were randomly assigned to two subplots of five randomly selected whole plots. A total of 390 test genotypes (including 120 flax accessions used for analysis in this paper and 270 breeding lines which varied in different years) were randomly allocated to the remaining subplots (test plots). Usually each test genotype appeared only once. The same design layout was applied to field experiments in three years and at two locations.

OIL and LIN revealed considerable differences among years (Y), locations (L) and their interaction (Y × L) (Table 4), showing substantial environmental differences during flax growing seasons between Saskatoon and Morden in 2009-2011. Significant genetic variation among the 120 flax genotypes was observed, although significant interactions also existed between genotypes (G) and years (G × Y), between genotypes and locations (G × L) and among all three factors (G × Y × L). When we summed up all the MS of genotype related terms, G, G × Y, G × L and G × Y × L (Table 4) and calculated the percentage of variation due to genotypes and interactions, we noticed that the variation due to genotypes accounted for 65% for yield, 85% for OIL and 94% for LIN. Interactions between genotype and environment constitute therefore only a small part of the genetic variation and yield is more readily affected by the environment than LIN and OIL. The Spearman rank-order and Pearson correlation coefficients between different experiments (Table 5) further confirmed this finding. Significant correlations were observed between any two experiments during three years at two locations for all three traits with the highest correlations in LIN and OIL.

Evaluation of test genotypes

Joint analysis over multiple environments permits the performance evaluation of test genotypes and their stability. We calculated the seed yield mean and CV of all test genotypes over 2 years and 2 locations for yield and drew a plot of means vs. CVs (Fig 2). The plot control 'CDC Bethune' was used as a check to draw a vertical line of CV and a horizontal line of mean yield. The genotypes with higher yield and smaller CV than the check were deemed stable and high yielding. A total of 24 of the 120 genotypes fell into this category, many of which are current cultivars, such as PrairieGrande, Hanley, Lirina, PrairieThunder and CDCMons. When the criteria of means and CV were slightly relaxed (in the dashed oval in Fig 2), most cultivars of Western Canada were included. As such, mean and CV over multiple environments are two indicators of yield selection and germplasm evaluation.

Compared with OIL and LIN, seed yield varied substantially more in different environments (Table 6). The average CV of the 120 genotypes for yield was approximately ten times larger than that of OIL and LIN, indicating that seed yield is largely affected by the environment. As such, seed yield phenotyping necessitates comprehensive evaluation in different environments, but for LIN and OIL, one or few environments may be sufficient for early selection and genetic study.

Discussion

Because of limited seed supply of test lines in the early stage of breeding scheme, insufficient seeds are available for a replicated experiment. In addition, for large numbers of test lines, it is difficult to arrange them in one block because of environmental heterogeneity in the field. Thus the augmented design with only one replication for test lines was proposed (Federer, 1956; Federer et al., 1975; Federer and Raghavarao, 1975). The basic idea of the augmented design is that (1) control lines are arranged in a standard design; (2) each replication of the control lines is placed in a soil-homogeneous block, and (3) the block is augmented to contain more non-replicated test lines. Based on control lines in a standard design, the block effects can be estimated to adjust the observed values of the test lines, and the error to test the significance of performance differences among lines. However, two problems arise in implementation of such an

Table 2. Unadjusted and adjusted values of seed yield (g m^{-2}) of subplot controls and their ANOVA results in the 2010/SK experiment. Adjustment by M1, M3 or M1+M3 obviously reduced soil heterogeneity represented by differences between means of plots and MS between plots in each adjustment method, but did not change MS between controls and MS of subplot error (retaining the same values for all adjustment methods), showing no relationship between adjustment efficiency and subplot error.

Plot (Row, column)	Control	Unadj.	M1	M3	M1+M3
3,1	Control A	23.80	25.53	24.15	24.44
4,3	Control A	26.67	25.36	24.86	24.42
4,4	Control A	26.23	26.40	24.75	24.56
4,10	Control A	25.71	24.69	24.62	24.32
7,8	Control A	24.84	24.56	24.41	24.31
3,1	Control B	20.90	22.63	21.25	21.54
4,3	Control B	26.63	25.32	24.82	24.38
4,4	Control B	25.67	25.84	24.19	24.00
4,10	Control B	25.11	24.09	24.02	23.72
7,8	Control B	24.24	23.96	23.81	23.71
3,1	Control C	22.73	24.46	23.08	23.37
4,3	Control C	25.35	24.04	23.54	23.10
4,4	Control C	24.24	24.41	22.76	22.57
4,10	Control C	22.89	21.87	21.80	21.50
7,8	Control C	25.79	25.51	25.36	25.26
Mean of Control A		25.45	25.31	24.56	24.41
Mean of Control B		24.51	24.37	23.62	23.47
Mean of Control C		24.20	24.06	23.31	23.16
Mean of Plot 3,1		22.48	24.21	22.83	23.12
Mean of Plot 4,3		26.22	24.91	24.41	23.97
Mean of Plot 4,4		25.38	25.55	23.90	23.71
Mean of Plot 4,10		24.57	23.55	23.48	23.18
Mean of Plot 7,8		24.96	24.68	24.53	24.43
MS between plots (DF=4)		5.84	1.69	1.46	0.91
MS between controls (DF=2)		2.12	2.12	2.12	2.12
MS of subplot error (DF=8)		1.12	1.12	1.12	1.12

DF: degrees of freedom; MS: mean square.

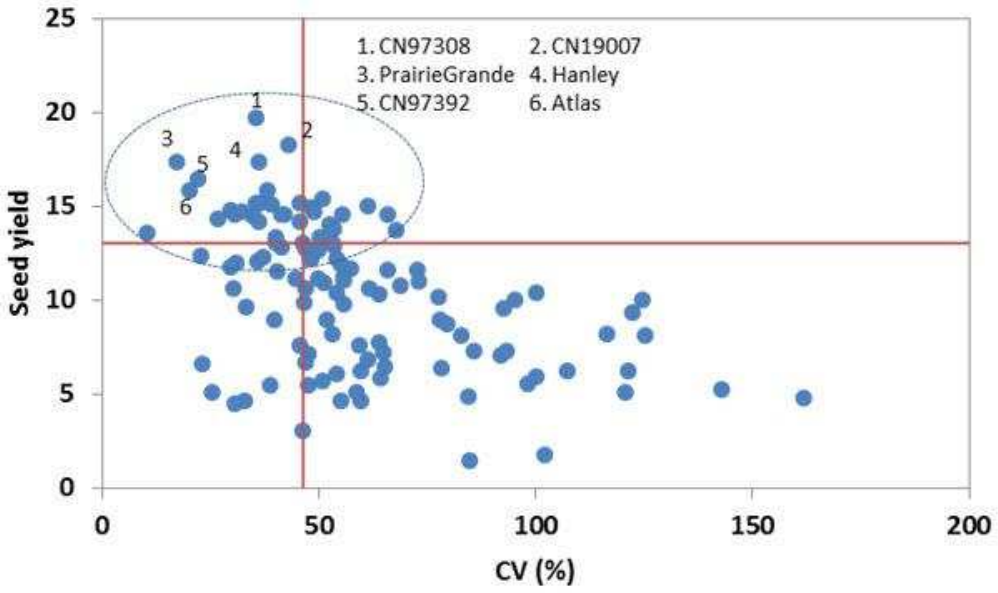

Fig 2. Plot of means vs. CVs over four environments for seed yield. The vertical and horizontal lines represent the CV and mean of the plot control cultivar, 'CDC Bethune' (Control A), respectively. Genotypes on the top-left quadrant are those with higher yield and lower CV than the plot control. The genotypes in the dashed oval include almost all current cultivars.

augmented design (Lin and Poushinsky, 1983). First of all, the control lines are assigned randomly to the plots in a block, resulting in their irregular distribution pattern over the experiment field. Though this placement gives unbiased error estimates, it is a disadvantage compared to systematic placement when adjusting for soil heterogeneity, particularly when control plots are used as soil fertility indicators. This is because the major purpose of the early stage selection experiments is to estimate the genotypic values of test lines, rather than to test the line difference critically. Second, the arrangement of test plots and control plots within a block is random, and the shape of a block is undefined (Lin and Poushinsky, 1983). For the large number of test lines in a block, arranging test plots and control plots within the block

Table 3. Coefficients of variation (CV) of unadjusted (Unadj.) and adjusted values by Method 1 (M1) and Method 3 (M3) for plot and subplot controls and relative efficiencies (RE) measured by error variance of all controls

Experiment	Trait	CV (%) for plot controls				CV (%) for subplot controls				RE			Best adjustment by	
		Unadj.	M1	M3	M1+M3	Unadj.	M1	M3	M1+M3	M1	M3	M1+M3	RE	ANOVA
2010/MD	Yield	21.1	15.8	6.1	11.1	8.1	11.2	9.2	10.7	172	1,042	337	M3	M1+M3
2010/SK	Yield	27.8	19.3	26.0	16.4	31.2	26.9	31.1	27.6	203	114	272	M1+M3	M1+M3
2011/MD	Yield	22.4	20.7	17.8	16.5	16.8	20.8	17.6	21.3	110	153	164	M1+M3	Un.
2011/SK	Yield	9.0	6.6	2.2	1.4	7.1	5.1	5.4	5.1	184	1,138	2,109	M1+M3	M1+M3
2009/MD	OIL	1.4	1.1	0.2	0.4	3.8	3.6	3.8	3.8	165	1,473	753	M3	M1+M3
2009/SK	OIL	0.7	0.6	0.6	0.1	3.3	3.3	3.3	3.3	137	160	1,093	M1+M3	M1+M3
2010/MD	OIL	2.7	1.9	2.4	2.0	4.4	3.9	4.3	3.9	212	132	185	M1	M1
2010/SK	OIL	2.1	1.6	0.3	0.0	2.9	2.7	2.4	2.3	162	2,917	14,567	M1+M3	M1+M3
2011/MD	OIL	1.4	1.3	0.8	0.8	4.3	4.3	4.4	4.4	116	239	278	M1+M3	Un.
2011/SK	OIL	0.8	0.7	0.3	0.1	3.7	3.6	3.7	3.7	152	605	1,064	M1+M3	M1+M3
2009/MD	LIN	2.1	1.4	1.1	1.4	1.2	1.4	1.1	1.4	206	364	224	M3	M1+M3
2009/SK	LIN	1.4	1.2	0.9	0.9	1.1	1.1	1.0	1.0	118	204	219	M1+M3	Un.
2010/MD	LIN	3.3	1.5	1.0	2.1	2.3	1.6	1.5	1.5	481	932	250	M3	M1
2010/SK	LIN	1.0	0.9	0.2	0.6	1.2	1.3	0.9	1.1	115	1,298	221	M3	Un.
2011/MD	LIN	1.5	1.3	2.1	1.3	1.1	1.7	1.4	1.7	122	50	115	M1	M1
2011/SK	LIN	1.1	0.9	1.5	1.0	1.5	1.6	2.9	2.5	143	40	82	M1	M1

Un.: unnecessary. The best adjustment by ANOVA was based on significance of mean squares (MS) and comparison of MS for M1, M3 and M1+M3. Here if both the row and column effects and the R × C interaction are not significant statistically, no adjustment is considered necessary.

Table 4. Joint ANOVA of seed yield, OIL, IOD and LIN with the type 2 MAD over two or three years and two locations.

Source	Yield (g m^{-2}) †			OIL (%)			LIN (%)		
	DF	MS	F	DF	MS	F	DF	MS	F
Year (Y)	1	1,526.23	423.90**	2	252.29	827.77**	2	540.84	1,239.05**
Location (L)	1	1,504.57	417.94**	1	403.30	1,300.97**	1	5,488.60	12,474.09**
Y × L	1	5,509.23	1,530.15**	2	152.69	500.97**	2	987.67	2,246.30**
Genotype (G)	119	91.48	25.41**	119	33.89	103.32**	119	344.47	782.89**
G × Y	119	20.75	5.76**	220	1.96	6.43**	220	8.96	20.38**
G × L	118	12.29	3.41**	119	2.17	7.00**	119	7.27	16.52**
G × Y × L	116	15.43	4.29**	200	1.65	5.42**	212	6.25	14.21**
Error	430	3.60		646	0.31		646	0.44	

** represents significance at the 1% probability level. †Data of two years and two locations were used for yield. MS: mean square; DF: degrees of freedom. The DF of error (430) is greater than $yl((rc-1)+2(m-1))=428$ (see Table 9) because two subplot controls were also assigned to test plots.

is critical for enhancing the efficiency of adjustment for soil heterogeneity. These problems were taken into account in the MAD. The MAD has a similar design structure to the augmented design but the control and test lines are systematically allocated within a block to enhance adjustment for soil heterogeneity. Two sub-types of MAD have been proposed (Lin and Poushinsky, 1983, 1985). The type 1 MAD is used for square plots (Lin and Poushinsky, 1983) while the type 2 MAD for rectangular plots (Lin and Poushinsky, 1985).

All previous studies of the type 2 MAD aimed to adjust for soil heterogeneity using two methods (M1 and M3) proposed by Lin and Poushinsky (1983, 1985) and Lin and Voldeng (1989) and then to verify the selected method using RE which was estimated based on subplot controls. The limited number of subplot controls reduces the precision of the RE estimates. Our results showed that when the number of whole plots harboring two subplot controls is small (only 5 in this study), the RE estimates based on subplot controls alone were not able to account for the change of soil variation after adjustment (Table 2). Subsequently, we proposed a different approach to assess the efficiency of the MAD, involving a comparison of the ANOVA results of unadjusted and adjusted data. We found that either of M1 or M3 alone failed to sufficiently eliminate soil variation in both the row and column directions and the R × C interaction. A combined method of M1+M3 has been proposed to adjust for soil variation from different directions, especially when both the row and column and the R × C interaction effects are significant. Furthermore, we redefined RE based on a variance ratio of unadjusted and adjusted values within both plot and subplot controls. This redefined RE can be used as an indicator of adjustment efficiency. In most cases, the combined method of M1+M3 was found to be superior.

In the type 2 MAD, subplot controls have two roles, estimating subplot error to test if the R × C interaction effect is statistically significant, and, estimating RE to verify the adjustment method suggested by the ANOVA. Both roles necessitate a sufficient sample size, i.e., the number of randomly selected whole plots for subplot controls, to have an adequate degree of freedom and power for the statistical test of the R × C interaction as well as to correctly estimate random error. Nevertheless, because the RE was redefined in this study based on both plot and subplot controls, it was able to sensitively indicate a suitable adjustment method, specifically M1+M3, to eliminate both row and column effects and the R × C interaction effects even through some significant R × C interaction effects were possibly not detected due to less test power. Thus, the redefined RE can compensate to some extent for the deficiencies associated with a reduced number of subplot controls.

To date the MAD is mostly used for early selection of breeding lines. In genomics study, QTL identification, association mapping and genomic selection necessitate phenotyping of yield and other traits of agronomic and economic importance of a large number of individuals in a mapping population. Though these individuals may have adequate amount of seeds for replicated experiments, homogeneous blocks in a field to accommodate large numbers of genotypes are still required. If soil homogeneity in blocks cannot be met, the MAD would be a good choice. The results in this study indicated that soil heterogeneity can be sufficiently adjusted for all traits in all experiments, suggesting that the MAD can be used for phenotypic analysis of any crop germplasm to obtain genetic value estimates of traits. In addition, yield is readily affected by environment and phenotyping of low heritability traits like yield needs multiple environments to assess stability or response of traits to environmental changes. For traits of high heritability like oil content or fatty acid compositions which have a relatively simple genetic control (Banik et al., 2011), one or few environments may be sufficient for their phenotyping.

A computer program package "MADPipeline" has been developed in this study for automated statistical analysis of MAD data with SAS software and Perl scripts available at http://probes.pw.usda.gov/bioinformatics_tools/ MADPipeline/index.html. The program facilitates data analysis of an unlimited number of experiments with any number of traits without customization of the program and human interference. A suitable adjustment of the observed values is automatically performed based on the following two rules: (1) if the MS of a row and a column is not greater than that of the R × C interaction, and the MS of the R × C interaction is not greater than that of the subplot error, the soil variation is considered homogeneous and no adjustment is necessary; (2) if the MS of either a row or a column is greater than that of the R × C interaction, or the MS of the R × C interaction is greater than that of the subplot error, the best adjustment method will be chosen based on the RE of three adjustment methods: M1, M3 and M1+M3.

Materials and methods

Plant materials

A collection of 120 accessions randomly selected from the flax core collection (Soto-Cerda et al., 2013) was used for evaluation.

Experimental design

A type 2 MAD with a 10 rows by 10 columns Latin Square was used to accommodate the 120 flax accessions, and other breeding lines used in mainstream flax breeding programs (Fig 1). Each whole plot was 2 m long and 2 m wide and was split into 5 subplots. The plot control cultivar 'CDC Bethune' (Control A) was placed in the center subplot of each whole plot. Two additional subplot control cultivars, 'Macbeth' (Control B) and 'Hanley' (Control C), were randomly assigned to subplots of each of 5 randomly selected whole plots. Thus, this design contained 100 whole plots with a total of 500 subplots, accommodating one plot control (Control A) in 100 central subplots, two subplot controls (Control B and C) in 10 subplots of 5 whole plots, and 390 test lines in the remaining subplots, accounting for 78% of all subplots. The design and assignment of test lines were carried out using the Agrobase software V.34.2.1 (Agronomix Software Inc, Winnipeg, Canada).

The same design with the same control cultivars was applied to individual experiments in three years (2009, 2010 and 2012) and two locations, Morden in Manitoba (MD) and Saskatoon in Saskatchewan (SK). The same collection of 120 flax genotypes was randomly allocated to subplots for each environment (year or location) except that 17 genotypes were missing at SK in 2009 and 16 at MD, also in 2009.

The seed yield was recorded in each experiment by harvesting two representative half meter (50 cm) sections within each subplot. Oil content was measured by NMR as outlined in FOSFA (Federation of Oils, Seeds and Fats Associations Limited) extraction method (Diederichsen et al., 2006). Fatty acid profiles of all test genotypes were obtained by gas chromatography (Varian 3800, Varian Analytical Instruments, Mississauga, Ontario, Canada) based on fatty acids methyl esters (FAMEs) extracted from seeds according

Table 5. Spearman rank-order (upper-right) and Pearson (lower-left) correlation coefficients between different experiments for yield, OIL and LIN. The diagonals are means and standard deviations of three traits over all test genotypes.

Experiment	Trait	2009/MD	2009/SK	2010/MD	2010/SK	2011/MD	2011/SK
2009/MD	OIL (%)	43.2±2.5	0.77**	0.72**	0.47**	0.64**	0.77**
	LIN (%)	54.2±4.0	0.79**	0.77**	0.80**	0.69**	0.79**
2009/SK	OIL (%)	0.80**	44.6±2.5	0.63**	0.63**	0.62**	0.82**
	LIN (%)	0.86**	56.5±3.4	0.71**	0.69**	0.76**	0.83**
2010/MD	Yield (g m^{-2})	-	-	10.4±4.9	0.47**	0.79**	0.66**
	OIL (%)	0.72**	0.68**	42.2±2.4	0.57**	0.72**	0.67**
	LIN (%)	0.83**	0.80**	50.5±10.0	0.74**	0.78**	0.82**
2010/SK	Yield (g m^{-2})	-	-	0.47**	7.2±3.8	0.39**	0.33**
	OIL (%)	0.53**	0.68**	0.69**	42.4±2.6	0.53**	0.60**
	LIN (%)	0.82**	0.78**	0.94**	61.0±10.3	0.67**	0.79**
2011/MD	Yield (g m^{-2})	-	-	0.76**	0.39**	6.8±4.2	0.63**
	OIL (%)	0.72**	0.69**	0.79**	0.66**	40.8±2.3	0.66**
	LIN (%)	0.76**	0.80**	0.94**	0.92**	50.5±8.7	0.82**
2011/SK	Yield (g m^{-2})	-	-	0.65**	0.29**	0.59**	17.2±5.9
	OIL (%)	0.80**	0.83**	0.72**	0.65**	0.70**	44.2±2.6
	LIN (%)	0.84**	0.87**	0.87**	0.84**	0.87**	55.0±8.6

** represents significance at the 1% probability level.

Table 6. Basic statistics of mean ($\overline{X}$) and coefficient of variation (*CV*) of test genotypes.

Trait	$\overline{\overline{X}}$ ± SD	$\overline{CV}$ ± SD
Yield (g m^{-2})†	9.33 ± 3.78	65.45 ± 21.71
OIL (%)	42.94 ± 2.22	4.41 ± 1.67
LIN (%)	55.76 ± 4.01	7.77 ± 2.09

SD: standard deviation. † Data from only four environments. $\overline{X}$ is the mean of each genotype across four or six environments. $\overline{\overline{X}}$ is the mean of all test genotypes. *CV* is the coefficient of variation of each genotype across four or six environments. $\overline{CV}$ is the mean of all test genotypes.

to AOAC method 996.06 (Daun et al., 1983; Association of Official Analytical Chemists, 2001). In this paper, only oil content (OIL), linolenic acid (LIN) and seed yield were used as an example for the proof-of-principle analysis.

ANOVA of plot and subplot controls in individual experiments

The purposes of the ANOVA of individual type 2 MAD experiments are to determine whether there is soil heterogeneity and to characterize its property, i.e., additive soil variation in a row or column direction, or non-additive variation in multiple directions, and to subsequently adjust observations of test genotypes to eliminate error effects due to soil heterogeneity. The basic idea is to use plot controls in the row and column design for estimating the plot error as well as the row and column effects, and subplot controls for estimating subplot errors. The plot error tests the null hypothesis of no significant differences of the plot controls in the rows and columns, i.e., there is no soil heterogeneity in the row or column direction. The subplot error tests whether the R × C interaction (plot error) is statistically significant. If a significant R × C interaction is detected, the soil variation then is considered non-additive, i.e., due to multiple directions. The test results determine if the observations of test genotypes necessitate adjustment and what method should be adopted (Lin and Poushinsky 1983; Lin and Poushinsky 1985).

A linear model of effects of the plot control can be written as follows:

$$x_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij} \quad (1)$$

where x_{ij} is the observed value of the plot control at the ij^{th} whole plot (i = 1, 2, …, r; j = 1,2,…, c); μ is the overall population mean of the plot control cultivar; α_i is the row effect at the i^{th} row; β_j is the column effect at the j^{th} column; and ε_{ij} is the plot error.

Similarly, a linear model of effects of the subplot controls can be described as follows:

$$x_{ij} = \mu + \varphi_i + v_j + \varepsilon_{ij} \quad (2)$$

where x_{ij} is the observed value of the subplot control at the i^{th} plot (i = 1, 2, …, m) and the j^{th} subplot (j = 1, 2, …, n); μ is the overall mean of subplot controls; φ_i is the plot effect at the i^{th} plot; v_j is the control effect of the j^{th} subplot control within the i^{th} plot; and ε_{ij} is the subplot error. The ANOVA of the subplot controls is equivalent to that of a RCBD with m blocks/replications (plots here) and n subplot controls in each block/plot. In order to increase the test power of the ANOVA, the plot control cultivar is also included as a subplot control, and therefore there are three subplot controls for analysis (n=3) (Lin and Voldeng, 1989). The expected mean squares and F tests are listed in Table 7. It is notable that the DF of subplot error should be (m-1)(n-1) not m(n-1) as per the paper of Lin and Voldeng (1989). If the DF of subplot error is m(n-1), then the subplot error will confound the variance among m plots (DF=m-1), resulting in overestimating the error, and subsequently decreasing the power to detect R × C effects. The correct ANOVA of subplot controls should consist of three variance sources: between plots, between controls and subplot error as shown in Table 7.

Adjustment of observed values for test genotypes

Three adjustment methods were proposed for the MAD (Lin and Poushinsky, 1983, 1985), in which Method 1 and Method 3 were evaluated to be suitable methods for the type 2 MAD (Lin and Voldeng, 1989; Casler et al., 2000). If the row or column effect is significantly larger than the plot error, the means of the plot controls of rows and columns are used to adjust the observed values of the test genotypes (Method 1). Let x_{ij} be an observed value of the plot control at the i^{th} row and the j^{th} column, the adjusted value (y'_{ijk}) is

$$y'_{ijk} = y_{ijk} - \alpha_i - \beta_j = y_{ijk} - \overline{X}_{i.} - \overline{X}_{.j} + 2\overline{X}_{..} \quad (3)$$

where y_{ijk} is the observed value at the k^{th} subplot (test genotype) of the ij^{th} whole plot; $\overline{X}_{i.}$ is the mean of the plot controls in the i^{th} row with $\overline{X}_{i.} = \sum x_{i.} /c$; $\overline{X}_{.j}$ is the mean of the plot controls in the j^{th} column with $\overline{X}_{.j} = \sum x_{.j} /r$; $\overline{X}_{..}$ is the overall mean of the plot controls with $\overline{X}_{..} = \sum x_{ij} /(rc)$; α_i is the row effect in the i^{th} row with $\alpha_i = \overline{X}_{i.} - \overline{X}_{..}$; β_j is the column effect in the j^{th} column with $\beta_j = \overline{X}_{.j} - \overline{X}_{..}$.

If the R × C interaction (plot error) is significantly larger than the subplot error, a regression method can be used for adjustment (Method 3):

$$y'_{ijk} = y_{ijk} - b(x_{ij} - \overline{X}_{..}) \quad (4)$$

where b is the regression coefficient of the mean of four test genotypes or test genotypes plus subplot controls on the values of plot controls in all whole plots (Lin and Voldeng, 1989).

When both the row and column effects and the R × C interaction are statistically significant, a combined method of Method 1 and Method 3 is proposed here to adjust for this complicated soil variation by applying eq. (3) followed by eq. (4). The b, x_{ij} and $\overline{X}_{..}$ in eq. (4) are calculated based on the values adjusted by Method 1.

To verify the efficiency of an adjustment method, the relative efficiency (RE) was defined as the ratio of the variance (pooled mean squares within controls) based on the unadjusted and adjusted data of subplot controls (Lin and Poushinsky, 1985; Lin and Voldeng, 1989). Here we redefined RE as a ratio of pooled variance within both plot and subplot controls of the unadjusted values to that of adjusted values:

$$RE = \frac{MS_{unadj}}{MS_{adj.}} \times 100 \quad (5)$$

Where, $MS = \frac{\sum\sum y_{ij}^2 - \sum(n_i \overline{Y}_i^2)}{\sum n_i - k}$, y_{ij} is the adjusted or unadjusted values of the j^{th} replicated value in the i^{th} control, $\overline{Y}_i$ is the mean of the i^{th} control, k is the number of controls (here k=3) and n_i is the number of replicated values of the i^{th} control.

Joint statistical analysis of multi-environmental experiments

For field experiments with the same design over multiple environments (years and locations), joint statistical analysis of adjusted values can be carried out to test the stability of

Table 7. ANOVA of the type 2 MAD design at a single location.

Source of variation	DF	MS	F test	EMS
Analysis of plot control				
Rows (R)	r-1	MS_R	MS_R/MS_{E2}	$\sigma_1^2 + \sigma_2^2 + c\sigma_R^2$
Columns (C)	c-1	MS_C	MS_C/MS_{E2}	$\sigma_1^2 + \sigma_2^2 + r\sigma_C^2$
Plot error (R × C)	(r-1)(c-1)	MS_{E2}	MS_{E2}/MS_{E1}	$\sigma_1^2 + \sigma_2^2$
Analysis of subplot controls				
Plots	m -1	MS_P	MS_P/MS_{E1}	$\sigma_1^2 + n\sigma_P^2$
Controls	n -1	MS_{Co}	MS_{Co}/MS_{E1}	$\sigma_1^2 + m\kappa_{CO}^2$
Subplot error	(n-1)(m-1)	MS_{E1}		σ_1^2

DF: degree of freedom; MS: mean square; EMS: expected mean square; n: two subplot controls plus one plot control, thus n=3; m: the number of whole plots randomly selected for subplot controls, here m=5; $\kappa_{CO}^2 = \dfrac{\sum \nu_i^2}{n-1}$.

Table 8. Joint ANOVA of the type 2 MAD at multiple locations.

Source of variation	DF	MS	F test	EMS (fixed model)
Location (L)	l-1	MS_L	MS_L/MS_E	$\sigma_E^2 + g\kappa_L^2$
Genotype (G)	g-1	MS_G	MS_G/MS_E	$\sigma_E^2 + l\kappa_G^2$
G × L	(g-1)(l-1)	MS_{GL}	MS_{GL}/MS_E	$\sigma_E^2 + \kappa_{GL}^2$
Error	ln_c	MS_E		σ_E^2

$n_c = (rc - 1) + 2(m-1)$, where rc is the number of adjusted values of the plot control, m is the number of whole plots selected for subplot controls (see Table 7). $\kappa_G^2 = \dfrac{\sum \tau_i^2}{g-1}$, $\kappa_L^2 = \dfrac{\sum \upsilon_j^2}{l-1}$, and $\kappa_{GL}^2 = \dfrac{\sum (\tau\upsilon)_{ij}^2}{(g-1)(l-1)}$.

Table 9. ANOVA of the modified augmented design over multiple years and locations.

Source of variation	DF	MS	Model 1 (fixed model)		Model 2 (mixed model, L and G fixed, Y random)	
			F test	EMS	F test	EMS
Year (Y)	y-1	MS_Y	MS_Y/MS_E	$\sigma_E^2 + gl\kappa_Y^2$	MS_Y/MS_E	$\sigma_E^2 + gl\sigma_Y^2$
Location (L)	l-1	MS_L	MS_L/MS_E	$\sigma_E^2 + gy\kappa_L^2$	MS_L/MS_{YL}	$\sigma_E^2 + g\sigma_{YL}^2 + gy\kappa_L^2$
Y × L	(y-1)(l-1)	MS_{YL}	MS_{YL}/MS_E	$\sigma_E^2 + g\kappa_{YL}^2$	MS_{YL}/MS_E	$\sigma_E^2 + g\sigma_{YL}^2$
Genotypes (G)	g-1	MS_G	MS_G/MS_E	$\sigma_E^2 + yl\kappa_G^2$	MS_G/MS_{GY}	$\sigma_E^2 + l\sigma_{GY}^2 + yl\kappa_G^2$
G × Y	(g-1)(y-1)	MS_{GY}	MS_{GY}/MS_E	$\sigma_E^2 + l\kappa_{GY}^2$	MS_{GY}/MS_E	$\sigma_E^2 + l\sigma_{GY}^2$
G × L	(g-1)(l-1)	MS_{GL}	MS_{GL}/MS_E	$\sigma_E^2 + y\kappa_{GL}^2$	MS_{GL}/MS_{GYL}	$\sigma_E^2 + \sigma_{GYL}^2 + y\kappa_{GL}^2$
G × Y × L	(g-1)(y-1)(l-1)	MS_{GYL}	MS_{GYL}/MS_E	$\sigma_E^2 + \kappa_{GYL}^2$	MS_{GYL}/MS_E	$\sigma_E^2 + \sigma_{GYL}^2$
Error	yln_c	MS_E		σ_E^2		σ_E^2

MS_E is estimated based on all replicated data of 3 control cultivars. See Table 8 for n_c, κ_G^2, κ_L^2 and κ_{GL}^2. $\kappa_Y^2 = \frac{\sum \omega_i^2}{y-1}$, $\kappa_{GY}^2 = \frac{\sum (\tau\omega)_{ik}^2}{(g-1)(y-1)}$, $\kappa_{YL}^2 = \frac{\sum (\upsilon\omega)_{jk}^2}{(l-1)(y-1)}$ and $\kappa_{GYL}^2 = \frac{\sum (\tau\upsilon\omega)_{ijk}^2}{(g-1)(l-1)(y-1)}$.

yield and other traits of the test genotypes across different environments. Since there is no replication for test genotypes, the joint experimental error can be estimated based on the adjusted values of one plot control and two subplot controls.

Because soil heterogeneity of control plots has been eliminated by data adjustment, the *rc* adjusted values of the plot control can be considered replicated values. Similarly, two subplot controls also have *m* replicated values. Therefore, a joint one-way ANOVA with three cultivars and different replications of each cultivar can be used to estimate the experimental error in multiple environments.

For the ANOVA at multiple locations in a year, the linear model is

$$y_{ij} = \mu + \tau_i + v_j + (\tau v)_{ij} + \varepsilon_{ij} \quad (6)$$

where y_{ij} is the adjusted value of the i^{th} genotype (i = 1, 2, …, g) at the j^{th} location (j = 1, 2, …, l); μ is the overall mean; τ_i is the genotype effect of the i^{th} genotype; v_j is the location effect of the j^{th} location; $(\tau v)_{ij}$ is the interaction effect between the i^{th} genotype and the j^{th} location, and ε_{ij} is the joint experimental error estimated based on the joint ANOVA of three control cultivars. The ANOVA is shown in Table 8.

The joint ANOVA of multiple years at one location has a similar linear model and expected mean squares as the ANOVA at multiple locations where locations are replaced by years.

For the joint ANOVA in multiple locations and years, the linear model is

$$y_{ijk} = \mu + \tau_i + v_j + (\tau v)_{ij} + \omega_k + (\tau\omega)_{ik} + (v\omega)_{jk} + (\tau v\omega)_{ijk} + \varepsilon_{ijk} \quad (7)$$

where y_{ijkl} is the observation of the i^{th} genotype (i = 1, 2, …, g) at the j^{th} location (j = 1, 2, …, l) in the k^{th} year (k = 1, 2, …, y); μ is the overall mean; τ_i is the genotype effect; v_j is the location effect; ω_k is the year effect; $(\tau v)_{ij}$, $(\tau\omega)_{ik}$, $(v\omega)_{jk}$, and $(\tau v\omega)_{ijk}$ are the interaction effects between genotypes and locations, between genotypes and years, between locations and years and between all three factors, respectively; and ε_{ijk} is the experimental error estimated using the same method as in the ANOVA at multiple locations (Table 9). The composition of the expected mean squares for sources of variation depends on an effect model, which in turn determines the denominator of the F tests. Two effect models (fixed model and mixed model) are listed in Table 9.

Pipeline package "MADPipeline" using SAS and Perl

The ANOVA in Tables 7, 8 and 9 were implemented using SAS software. The generalized linear model (PROC GLM) in SAS was used to calculate DF and MS. A pipeline program package was developed for data preparation, ANOVA and generating result summary. A SAS program (MADPipeline_ Step1a.sas) was written to perform ANOVA of individual MAD experiments, in which two separate ANOVA for plot and subplot controls are performed. The outputs from this SAS program are used as input of a downstream Perl program (MADPipeline_Step1b.pl) to summarize the ANOVA results, adjust the observations of the test genotypes and controls, and estimate the RE of the different adjustment methods. Ultimately, a data file with adjusted values of the best adjustment method is exported for further analysis. The adjusted values of the test genotypes and controls are further used as input of the second SAS program, MADPipeline_Step2a.sas, to perform the joint ANOVA over multiple environments, if any. It is worth noting that in the PROC GLM, all effects are considered fixed even when the "RAMDOM" statement is used. The PROC GLM is not able to choose suitable MS terms for the F tests in the mixed model (Model 2 in Table 9). Thus, an additional Perl program (MADPipeline_Step2b.pl) was provided to calculate the correct F values and perform the test of significance. A complete analysis procedure was described in the user's guide of this pipeline program package. The program package, named "MADPipeline", and its user's guide is freely downloadable at: http://probes.pw.usda.gov/bioinformatics_tools/MADPipeline/index.html. In order to take advantage of open source statistic software R (http://www.r-project.org/), the R version of MADPipeline will be developed as the next step.

Conclusion

The results of the present study indicated that the soil heterogeneity of the experimental field can be assessed through an MAD and effects due to soil heterogeneity can be sufficiently eliminated by a suitable adjustment method, especially the combined M1 and M3 method. The MAD is applicable to screen breeding lines in the early stages of selection and to phenotype traits in any crop for genetic studies, such as QTL identification using association mapping strategy. The reported computer pipeline package provides an easy and automated way to analyze data from MAD experiments.

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