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Experimental strategies in carrying out VCU for tobacco crop I: plot design and size

F.H.R.B. Toledo[1], M.A.P. Ramalho[2], C.E. Pulcinelli[3] and A.T. Bruzi[4]

[1]Departamento de Genética, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, SP, Brasil
[2]Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil
[3]Companhia Souza Cruz, Rio Negro, PR, Brasil
[4]Departamento de Agricultura, Universidade Federal de Lavras, Lavras, MG, Brasil

Corresponding author: F.H.R.B. Toledo
E-mail: fernandohtoledo@gmail.com

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ABSTRACT. We aimed to establish standards for tobacco Valor de Cultivo e Uso (VCU) in Brazil. We obtained information regarding the size and design of plots of two varietal groups of tobacco (Virginia and Burley). Ten inbred lines of each varietal group were evaluated in a randomized complete block design with four replications. The plot contained 42 plants with six rows of seven columns each. For each experiment plant, considering the position of the respective plant in the plot (row and column) as a reference, cured leaf weight (g/plant), total sugar content (%), and total alkaloid content (%) were determined. The maximum curvature of the variations in coefficients was estimated. Trials with the number of plants per plot ranging from 2 to 41 were simulated. The use of a border was not justified because the interactions between inbred lines x position in the plots were never significant, showing that the behavior of the inbred lines coincided with the different positions. The plant performance varied according to the column position in the plot. To lessen the effect of this factor, the use of plots with more than one row is recommended. Experimental precision, evaluated by the CV%, increased with an increase in plot size; nevertheless, the maximum curvature of the variation coefficient method showed no expressive increase in precision if the number of plants was greater than seven. The result in identification of the best inbred line, in terms of the size of each plot, coincided with the maximum curvature method.

Key words: Field trial; Plot size; Monte Carlo simulation; Statistics; Plant breeding

INTRODUCTION

The VCU (Valor de Cultivo e Uso) field production trial is a requirement of the Ministry of Agriculture, Livestock and Food Supply, Ministério da Agricultura, Pecuária e do Abastecimento (MAPA) (Law No. 9456 of 04/25/1997), with a view toward the registration of new cultivars (Brasil, 1997). This registration is indispensable for the commercialization of seeds of any cultivar, under protection or otherwise. Standards for carrying out the VCU vary according to each species and are established by MAPA.

In the establishment of these standards, the background of specialists in experimentation with the crop and available references in the literature are used. In the case of tobacco, for which Brazil is the largest exporter worldwide, collecting around 3 billion dollars a year, standards for carrying out the VCU are still being established (da Silveira et al., 2010).

Various aspects must be considered in the experimental strategy of the VCUs, such as the size and design of the plots and the number of replications. In the literature, some reports are found related to the optimization of efficiency of the experiments in an attempt to find the most appropriate size of the plots (Zanon and Storck, 2000; Ramalho et al., 2012). The focus is always on minimizing error and maximizing the efficiency of the tests at the lowest possible cost (Parnaiba et al., 2009). In the case of tobacco crop, information regarding the size of experimental plots remains scarce, and there are no references found for tropical and subtropical conditions.

Until recently, some companies used plots with six rows each and seven plants per row, in which the two outer rows and the plants at the extremities of the central rows were used as a border (Crew and Jones, 1963; Gupton, 1972). The key questions are whether there is the need for a border, and how many plants per plot are needed and which would be the most recommended disposal in order to obtain reliable estimates of the mean values of the cultivars under evaluation. This information for carrying out experiments varies according to the varietal group of the tobacco cultivar and the trait under evaluation.

Given the above information, the purpose of this study was to obtain information regarding strategies for the conduction of VCU experiments, using the Burley and Virginia varietal group tobacco crops, in relation to the size and design of the plots and number of replications.

MATERIAL AND METHODS

Experiments were installed for the evaluation of inbred lines of the Burley and Virginia varietal groups in the 2009/2010 growing season and conducted in the Rio Negro county, located in the state of Paraná, Brazil, at an altitude of 780 m, latitude 26°06'S and longitude 49°47'W. A randomized complete block design with four replications was used, consisting of 10 inbred lines in the final stage of evaluation, with plots of six rows with seven plants each, for a total of 42 plants per plot.

For each plant of the experiments, taking the position of the respective plant in the plot (row and column) as a reference, the cured leaf weight (g/plant), total sugar content (%), and total alkaloid content (%) were obtained. Variance analysis was carried out using three alternatives: considering the useful area or border area; considering the arrangement in rows or columns; and the useful area and total area. For this analysis, a procedure similar to that presented by Steel et al. (1997) was used, according to the following model: $y_{ijk} = \mu + r_i + t_j + (tr)_{ij} + s_k + (rs)_{ik} + (ts)_{ik} + \varepsilon_{ijk}$, where y_{ijk} is the observation in replication i, of treatment j, in position k; μ is the constant that, through the imposed restriction, is the mean of the observations; r_i is the effect of the replication i, with i = 1, 2, ..., 4; t_j is the effect of treatment j, with j = 1, 2, ..., 10; $(tr)_{ij}$ is the error term (a) among plots; s_k is the effect of position, regarding the position in the plot, according to the model used, between location, between rows, or columns of the plot; $(rs)_{ik}$ is the error term (b) of the subplot, since there was not randomization of the subplots in the plot; $(ts)_{ik}$ is the effect of the interaction of treatment i on position k; and ε_{ijk} is the error term (c), with $\varepsilon \sim N(0, \sigma^2)$.

Estimates of the maximum curvature of the variation coefficient were obtained. To achieve this, the residues of the adjusted model were used to estimate the first-order autocorrelation coefficient (ρ). The point of maximum curvature of the variation coefficient was obtained according to the expression $X_0 = (10(2(1 - \rho^2)\, \sigma^2 Z)^{1/3})/Z$, presented by Parnaiba et al. (2009), where X_0 is the point of maximum curvature of the variation coefficient (CV%); ρ is the first-order spatial autocorrelation coefficient; σ^2 is the variance of experimental error; and Z is the mean value of the plot.

In addition, the different numbers of plants per plot were simulated, ranging from 2 to 41. The plants were sampled randomly without replacement. The process was repeated 2000 times for each size. The resampling method was used in the following manner: an algorithm considering the arrangement of randomized complete blocks over the data frame chose n plants from each plot, performed the variance analysis, and stored the following estimates: mean squared error among plots (V_e) and variance within plots (V_w); the three lines with the best performance in each experiment were identified. All analyses were performed and/or implemented using the software R (R Development Core Team, 2011).

RESULTS AND DISCUSSION

The summary of the variance analyses, considering the performance of the lines located at the border or in the useful area for the Burley and Virginia varietal groups, are presented in Table 1. Initially, on considering the cured leaf weight (g/plant), it was verified that in terms of significance of the F test, the results referring to the two varietal groups of tobacco and to the three variables were very similar. The F test was significant for the inbred line ($P \leq 0.07$) and position ($P \leq 0.01$) sources of variation, indicating that the inbred lines under evaluation differed, and the mean performance of the lines varied as a result of the position in the plot. As expected, the mean performance of the inbred lines at the border was greater than that obtained in the useful area.

Table 1. Summary (P values) of the variance analyses of inbred lines evaluation (Burley and Virginia tobacco varietal groups) for cured leaf weight (g/plant), sugar content (%), and total alkaloid content (%), considering the border and the useful area of the plot.

Summary of variance analyses	d.f.	Burley			Virginia		
		Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)	Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)
Replication	3	0.09	<0.01	0.05	<0.01	0.55	<0.01
Inbred lines (*L*)	9	0.07	0.58	<0.01	<0.01	0.84	<0.01
Error *a*	27						
Between columns (*P*)	1	<0.01	0.12	<0.01	<0.01	0.42	<0.01
Error *b*	3						
Interaction (*L* x *P*)	9	0.27	0.33	0.56	0.54	0.68	0.41
Error *c*	27						
General mean		282.29	1.68	3.97	249.36	5.68	2.26
Useful area		259.52	1.65	4.06	267.32	5.62	2.30
Border		302.96	1.71	3.89	229.38	5.75	2.20
Accuracy		0.72	-	0.86	0.96	-	0.92

Rio Negro, Paraná, 2009/2010.

What draws most attention, however, is that the inbred lines x position interaction was not significant. In other words, the behavior of the inbred lines coincided regardless of whether they were in the border or in the useful area. The results of analyses of variance for the total alkaloid content were quite similar to that reported previously for cured leaf weight. In the case of sugar content, there was no significant difference for any of the sources of variation.

Variance analyses were also carried out by considering the total area of the plot and only the useful area. It was observed that the estimate of accuracy, which may be used as a measurement of precision (Resende and Duarte, 2007), was very similar between the two situations. The comparison of means considering the total area or only the useful area showed that, in the case of the Burley group, the Scott and Knott (1974) test identified only one group for the cured leaf and sugar traits under both conditions, or total area, or only useful area. In the case of alkaloids, the correspondence in classification of the inbred lines was identical in the two situations. Considering the Virginia group, for the cured leaf and alkaloid traits, the Scott and Knott test identified a greater number of groups in the classification of the lines. Although the correspondence in their classification was not perfect, it was possible to identify the line with best performance in the two situations, for both traits (Table 2).

Table 2. Means obtained in variance analyses of inbred lines evaluation (Burley and Virginia tobacco varietal groups). Cured leaf weight (g/plant), sugar content (%), and total alkaloid content (%). With all plants of the border or only those of the useful area.

Inbred lines	Burley						Virginia					
	Cured leaves (g/plant)		Sugar (%)		Alkaloids (%)		Cured leaves (g/plant)		Sugar (%)		Alkaloids (%)	
	Border	Useful	Border	Useful	Border	Useful	Border	Useful	Border	Useful	Border	Useful
1	332.39^{A}*	281.30^{A}	1.69^{A}	1.68^{A}	3.24^{B}	3.55^{B}	274.77^{A}	247.79^{A}	5.73^{A}	5.94^{A}	2.33^{B}	2.20^{B}
2	262.10^{A}	243.62^{A}	1.54^{A}	1.52^{A}	3.51^{B}	3.45^{B}	256.14^{B}	208.22^{D}	5.76^{A}	5.84^{A}	1.97^{D}	2.00^{B}
3	281.82^{A}	232.75^{A}	2.09^{A}	1.95^{A}	4.48^{A}	4.51^{A}	227.07^{C}	199.78^{D}	5.47^{A}	5.82^{A}	2.13^{C}	2.15^{B}
4	323.69^{A}	274.94^{A}	1.76^{A}	1.71^{A}	3.92^{A}	4.36^{A}	273.88^{A}	237.09^{B}	5.07^{A}	4.94^{A}	2.47^{A}	2.37^{A}
5	291.82^{A}	257.62^{A}	1.66^{A}	1.64^{A}	3.58^{B}	3.75^{B}	257.25^{B}	222.61^{C}	5.99^{A}	6.28^{A}	2.33^{B}	2.20^{B}
6	301.31^{A}	254.19^{A}	1.93^{A}	1.90^{A}	4.23^{A}	4.49^{A}	252.53^{B}	220.88^{C}	4.95^{A}	4.99^{A}	2.34^{D}	2.17^{B}
7	380.80^{A}	324.19^{A}	1.84^{A}	1.69^{A}	4.11^{A}	4.16^{A}	276.32^{A}	237.85^{B}	5.06^{A}	5.91^{A}	2.18^{C}	2.00^{B}
8	359.60^{A}	282.19^{A}	1.86^{A}	1.57^{A}	3.63^{B}	3.88^{B}	297.72^{A}	255.43^{A}	6.57^{A}	6.06^{A}	2.54^{A}	2.45^{A}
9	294.49^{A}	259.44^{A}	1.26^{A}	1.31^{A}	4.05^{A}	4.06^{A}	279.07^{A}	240.62^{B}	5.30^{A}	5.48^{A}	2.23^{C}	2.12^{B}
10	201.59^{A}	184.44^{A}	1.44^{A}	1.56^{A}	4.17^{A}	4.42^{A}	280.02^{A}	222.28^{C}	6.41^{A}	6.16^{A}	2.52^{A}	2.41^{A}
$M.S._{error}$	4247.81	3359.42	0.24	0.2	0.21	0.16	282.54	115.83	1.5	2.35	0.02	0.02
Accuracy	0.77	0.61	0.12	-	0.81	0.86	0.90	0.95	-	-	0.91	0.89

*Means in the same column followed by the same letter belong to the same group by the Scott and Knott (1974) test with α = 0.10. Rio Negro, Paraná, 2009/2010.

To prove the existence or not of the lines x position interaction, variance analyses were undertaken by considering the position of the rows in the plot both in the "horizontal" and "vertical" directions, but in this case without considering the existence of a border (Tables 3 and 4). Likewise, the results were very similar between the two varietal groups of tobacco. Taking Virginia as a reference, it was observed that there were no differences in the average performance of the lines in the different rows of the plot. However, the average performance of the lines varied among columns. This implies that, under these experimental conditions, the heterogeneity of the soil was in the horizontal direction. Nevertheless, in both cases, the lines x position interaction was not significant; that is, the performance of the lines coincided regardless of the row or column used.

Table 3. Summary (P values) of the variance analyses of inbred lines evaluation (Burley and Virginia tobacco varietal groups). For cured leaf weight (g/plant), sugar content (%) and total alkaloid content (%), considering the different rows of the plot.

Summary of the variance analyses	d.f.	Burley			Virginia		
		Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)	Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)
Replication	3	0.08	<0.01	0.05	<0.01	0.55	<0.01
Inbred lines (*L*)	9	0.06	0.57	<0.01	<0.01	0.83	<0.01
Error *a*	27						
Between columns (*P*)	5	0.42	0.03	0.06	0.22	0.01	0.07
Error *b*	15						
Interaction (*L* x *P*)	45	0.86	0.86	0.04	0.36	0.59	0.26
Error *c*	135						

Rio Negro, Paraná, 2009/2010.

Table 4. Summary (P values) of the variance analyses of inbred lines evaluation (Burley and Virginia tobacco varietal groups). For cured leaf weight (g/plant), sugar content (%) and total alkaloid content (%), considering the different columns of the plot.

Summary of the variance analyses	d.f.	Burley			Virginia		
		Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)	Cured leaves (g/plant)	Sugar (%)	Alkaloids (%)
Replication	3	0.08	<0.01	0.05	<0.01	0.55	<0.01
Inbred lines (*L*)	9	0.06	0.57	<0.01	<0.01	0.83	<0.01
Error *a*	27						
Between columns (*P*)	6	<0.01	0.35	<0.01	<0.01	0.07	<0.01
Error *b*	18						
Interaction (*L* x *P*)	54	0.10	0.26	0.03	0.91	0.09	0.23
Error *c*	162						

Rio Negro, Paraná, 2009/2010.

One of the arguments for use of a border is the intergenotypic competition among plots; that is, the performance of an inbred line may be altered in terms of the inbred line situated in the adjacent plot. All indications are that this fact did not occur in this experiment. Nevertheless, to mitigate this competition without the use of a border, the best option was the use of plots with more than one row. Fehr (1987) observed that with two rows, the intergenotypic competition was reduced by half, and with three rows, the reduction was 66.7%.

Another important question is how many plants each plot should have, or what the plot size should be. To obtain this information, uniformity data are normally used (Parnaiba et al., 2009). Although it is greatly used, this type of experiment mainly evaluates the heterogeneity of the experimental area (Zanon and Storck, 2000) and considers only one treatment.

Thus, for VCUs, the uniformity test is not a good alternative, because the experiments are conducted on the farmers' properties, and evaluating the heterogeneity of the area is not justified. The use of experiments such as that designed in this study should be more informative.

One of the procedures used to gain information about the number of plants in the plot is the appraisement of different plot sizes. In this case, some parameter, such as an evaluation of precision, is estimated. A highly used alternative is the estimate of the variation coefficient (CV%). This procedure was adopted, and the point of maximum curvature of the CV% was estimated (Figure 1). We observed that the CV% reduced the increase in precision with increase in the number of plants per plot. Similar results have been reported for other crops (Chaves, 1985; Camacho et al., 2000). Nevertheless, the increase was asymptotic and, by the proposed procedure, the ideal number of plants should be close to seven.

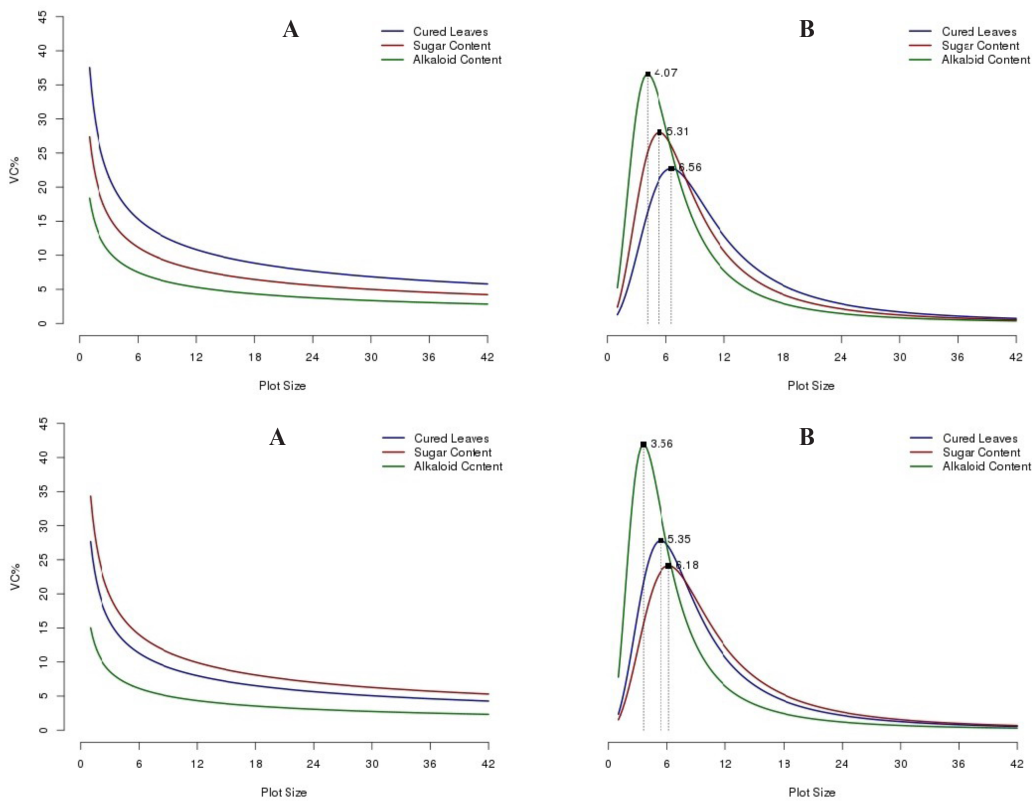

Figure 1. Curvature of the coefficient of variation (CV%) in terms of the plot size (A) and point of maximum curvature (B), for cured leaf weight (g/plant), sugar content (%), and alkaloid content (%), of the Burley (above) and Virginia (below) varietal groups. Rio Negro, Paraná, 2009/2010.

Considering that the breeder's interest in VCU is to identify the best inbred lines or lines to be recommended, another procedure would be to verify, in the 2000 simulations for each plot size, what percentage of these simulations identified the best inbred line, among the top three, in relation to the maximum size adopted. Tables 5 and 6 present the results for the

Burley and Virginia varietal groups, respectively, for the first inbred lines obtained in the analysis among the 42 plants in the plot. With regards the cured leaf weight in the Virginia varietal group, the coincidence of the best inbred line was already high, even when considering only three plants per plot. It was observed that with seven plants, the coincidence was greater than 90%; that is, when using seven plants chosen at random among the 42, in 91.15% of the 2000 simulations, the best treatment was among the three best.

Table 5. Percentage of coincidence between the classification of the best line of the Burley tobacco varietal group, for each plot size, with classification of the three best in the standard plot (42 plants). For cured leaf weight (g/plant), sugar content (%), and total alkaloid content (%).

Plot	Cured leaves (g/plant)		Sugar (%)		Alkaloids (%)	
2	0.86	(0.50)*	0.82	(0.55)	0.86	(0.45)
3	0.92	(0.59)	0.90	(0.63)	0.92	(0.52)
4	0.96	(0.69)	0.92	(0.66)	0.94	(0.57)
5	0.98	(0.73)	0.95	(0.71)	0.97	(0.63)
6	0.98	(0.75)	0.97	(0.73)	0.98	(0.67)
7	0.99	(0.79)	0.98	(0.74)	0.99	(0.70)
8	1.00	(0.83)	0.99	(0.76)	0.99	(0.72)
9	1.00	(0.84)	0.99	(0.79)	1.00	(0.76)
10	1.00	(0.88)	1.00	(0.80)	1.00	(0.79)
-	-	-	-	-	-	-
20	1.00	(0.98)	1.00	(0.92)	1.00	(0.95)
30	1.00	(1.00)	1.00	(0.99)	1.00	(1.00)
40	1.00	(1.00)	1.00	(1.00)	1.00	(1.00)
41	1.00	(1.00)	1.00	(1.00)	1.00	(1.00)

*Values between parentheses refer to coincidence with the best classified. Rio Negro, Paraná, 2009/2010.

Table 6. Percentage of coincidence between the classification of the best line of the Virginia tobacco varietal group, for each plot size, with classification of the three best in the standard plot (42 plants). For cured leaf weight (g/plant), sugar content (%) and total alkaloid content (%).

Plot	Cured leaves (g/plant)		Sugar (%)		Alkaloids (%)	
2	0.70	(0.39)*	0.64	(0.24)	0.87	(0.46)
3	0.76	(0.44)	0.71	(0.28)	0.92	(0.48)
4	0.82	(0.53)	0.74	(0.29)	0.95	(0.50)
5	0.85	(0.55)	0.80	(0.30)	0.97	(0.53)
6	0.89	(0.63)	0.83	(0.34)	0.98	(0.55)
7	0.91	(0.66)	0.85	(0.37)	0.99	(0.57)
8	0.92	(0.69)	0.87	(0.38)	1.00	(0.60)
9	0.94	(0.71)	0.89	(0.39)	1.00	(0.60)
10	0.95	(0.74)	0.91	(0.41)	1.00	(0.62)
-	-	-	-	-	-	-
20	1.00	(0.95)	0.99	(0.50)	1.00	(0.75)
30	1.00	(1.00)	1.00	(0.58)	1.00	(0.88)
40	1.00	(1.00)	1.00	(0.75)	1.00	(1.00)
41	1.00	(1.00)	1.00	(0.84)	1.00	(1.00)

*Values between parentheses refer to coincidence with the best classified. Rio Negro, Paraná, 2009/2010.

Finally, it could be questioned where the greatest variation in the tobacco experiments occurred; that is, among plants within the plot (V_w) or among plots that received the same treatment in the different repetitions (V_e). In this case, as lines were evaluated, the variation among plants within the plots was all environmental (Ramalho et al., 2012). Nevertheless, the V_e in some circumstances may also be due to deficient genetic sampling, which means that the plants of the plot might not represent the treatment, in this case the inbred lines. Thus, such deficiency would contribute to variations among the plots that received the same treatment in the different replications, and are not only due to the environment conditions.

The V_w/V_e ratio allows one to evaluate under which conditions the environmental variation is greater, and also if this ratio varies according to the number of plants in the plots. In Table 7, this ratio, estimated for a different number of plants per plot in the mean of the 2000 simulations, may be seen for the cured leaf trait of the Burley and Virginia groups, respectively. As observed, the ratio varied according to the varietal group. In general, the V_w/V_e ratio was greater in the Virginia varietal group. It should be noted that V_w was greater than V_e, giving a ratio greater than 1; that is, most of the variation responsible for experimental error occurred within the plots. This fact is consistently observed for other species grown, involving other traits (Souza and Ramalho, 1995; Moreto et al., 2007).

Table 7. Ratio between the variance within the plot V_w, and the variation of error among plot V_e, obtained for different numbers of plants; cured leaf weight (g/plant) trait in the Burley and Virginia varietal groups.

Plot	Burley		Virginia	
	V_w/V_e	$\sigma V_w/\sigma V_e^*$	V_w/V_e	$\sigma V_w/\sigma V_e$
2	3.72	0.62	7.46	0.65
3	3.79	0.38	7.83	0.48
4	3.88	0.28	7.79	0.35
5	3.81	0.22	7.90	0.30
6	3.83	0.19	7.83	0.26
7	3.88	0.15	7.67	0.22
8	3.81	0.14	7.74	0.20
9	3.84	0.12	7.85	0.17
10	3.80	0.11	7.90	0.16
-	-	-	-	-
20	3.80	0.05	7.83	0.09
30	3.81	0.04	7.83	0.06
40	3.82	0.03	7.83	0.05
41	3.82	0.03	7.83	0.04

*σ refers to standard deviation among the 2000 simulations. Rio Negro, Paraná, 2009/2010.

The main interest at this time was in verifying if this ratio varies according to the number of plants sampled in the plot. Taking the Burley group (Table 7) as a reference, the V_w/V_e ratio practically did not change according to the different number of plants evaluated, and, furthermore, this same tendency was observed for the ratio of the estimates of standard deviation of the variances observed in the 2000 simulations. These results corroborate the previous observations. Evaluating with regard to sampling, the variance among plants (V_w) or among plots (V_e) in terms of the number of plants per plot had little effect on the estimates obtained.

CONCLUSIONS

The use of a border is not justified, because the inbred lines x position interactions in the plots were never significant, showing that the behavior of the inbred lines coincided in the different positions.

Plant performance varied according to the column position in the plot. To lessen the effect of this factor, the use of plots with more than one row is recommended.

Experimental precision, evaluated by the CV%, increased along with the increase in plot size; nevertheless, the maximum curvature of the variation coefficient method showed that there are no expressive increases in precision if the number of plants is greater than seven.

The result in identification of the best inbred line, in terms of the size of each plot, coincided with the maximum curvature method.

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