

SHORT COMMUNICATION

Changes in N₂ fixation in *Stylosanthes scabra* derived from tissue culture

Maria José Valarini¹, Ivani Pozar Otsuk¹ and Maria Lucia Carneiro Vieira²

ABSTRACT

Plants were regenerated from leaf-derived callus culture of *Stylosanthes scabra*, a polyploid legume tolerant to drought and adapted to acid soils. A total of 168 regenerants were planted out in Leonard jars in a complete randomized design. Nitrogen fixation and vegetative growth were indirectly evaluated by shoot dry weight, root dry weight, shoot N content and acetylene reduction activity. The results showed higher variation in the regenerants than in controls not submitted to tissue culture. Significant differences were found for all nitrogen fixation related-traits.

INTRODUCTION

Tissue culture is clearly a mutagenic procedure (Phillips *et al.*, 1990). Although some tissue culture regenerants appear normal, a significant proportion (up to 30%) of plants may have altered characteristics not present in the controls. This type of frequent, unselected genetic variability detected in tissue culture regenerants has been termed somaclonal variation (Larkin and Scowcroft, 1981). Phillips *et al.* (1990) hypothesize that the various mutational events are related to the modification of DNA, specifically DNA hypo and hyper-methylation. These events include single gene mutations, transposable element activation, quantitative trait variation and chromosome rearrangements. Application of somaclonal variation approaches to crop improvement has been focused on selection for herbicide resistance, stress tolerance and disease resistance. There are few reports dealing with the evaluation of soma-

clones ability for nodulation and nitrogen fixation. The aim of the current study was to examine five attributes related to N₂ fixation and vegetative growth in regenerants of *Stylosanthes scabra* Vog., a tropical forage legume.

MATERIAL AND METHODS

Tissue culture

Seed-born plants of *Stylosanthes scabra* (EPAMIG Accession No. 1043) were cultivated axenically by seed surface sterilization in 70% (v/v) ethanol for 40 s followed by immersion in a 2% (v/v) NaOCl solution for 15 min. Seeds were rinsed four times in sterile water and placed in half-strength MS medium (Murashige and Skoog, 1962) containing 3.0% (w/v) of sucrose, free of hormones. Thirty days after seed sowing, expanded leaves were used as explants sources for tissue culture. The central part of the leaves was excised and placed on 0.9% (w/v) agar-solidified MS medium supplemented with 2.0 mg l⁻¹ BAP and 1.0 mg l⁻¹ NAA. After the callus phase (5 months), cultures were transferred to MS medium containing 2.0 mg l⁻¹

¹ Instituto de Zootecnia de Nova Odessa, Caixa Postal 60, 13460-000 Nova Odessa, SP.

² Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz"/USP, Caixa Postal 83, 13400-970 Piracicaba, SP, Brasil. Send correspondence to M.L.C.V.

BAP to induce shoot regeneration. Shoots were rooted and placed in half-strength MS medium containing 1.5% (w/v) of sucrose, free of hormones. Ten controls (plants not submitted to tissue culture) were obtained by cutting the stems of the 30-day old seedlings and transplanting them into the medium used for root induction. Both control and regenerants derived from the same pool of genotypes.

Plant assay

The 168 *S. scabra* plantlets used for this study were derived from 42 calli. The four replications were plantlets derived from the same callus and each callus was considered as parental genotype. Plants were individually grown in autoclaved Leonard jars containing a peat:sand mixture (2:1) and watered with Norris solution (Norris and Date, 1976). Jars were distributed in a complete randomized design with one plant per plot. After 7 days, the seedlings were inoculated with a liquid culture of *Bradyrhizobium* sp containing an equal mixture of 6155 SEMIA, 4969 CIAT and 1650 CB strains. The nodulation ability and nitrogen fixation were indirectly evaluated by shoot dry weight (SDW), root dry weight (RDW), shoot N content (N%, Total N) and acetylene reduction activity (ARA) by gas chromatography analysis, 90 days after inoculation.

Data analysis

Data were subjected to univariate analysis of variance for all traits. Means were compared by the Dunnett test. For data scoring it was used principal component analysis (Mardia et al., 1979) enabling the genotypes to be plotted on two axes. Cluster analysis was performed to all traits according to Bussab et al. (1990) by using the UPGMA method.

RESULTS AND DISCUSSION

The morphology of the 168 somaclones produced after a callus phase was highly variable. Most of them differed in appearance from the uniform group of control plants. Plants with modified architecture were very frequent, such as upright habit, increased branching and larger leaves. Changes in vigor were in both directions. Similar results were reported by Consoli et al. (1996) examining tissue culture effects on quantitative traits of *Stylosanthes guianensis*.

For all nodulation-related traits used in this study, means of the control and 42 batches of regenerants are presented in Table I. Data were subjected to

one-way analysis of variance and also to multivariate analysis. Normal distribution was tested and data transformed to \sqrt{x} . Transformed means were compared by the Dunnett test. Although tissue culture plants exhibited a broad range of variation just a few treatments were statistically different (Table I). Significant

Table I - Mean data for shoot dry weight (SDW), root dry weight (RDW), shoot N content (N%), acetylene reduction activity (ARA) and Total N of *Stylosanthes scabra* regenerants and controls (C). Means of tissue culture-derived plants were compared with those of the controls by the Dunnett test. Data are presented on the original scale. *Statistically different at 5% level of probability.

Genotype identification	SDW	RDW	N%	ARA	Total N
C	0.57	0.19	1.71	2306	8.79
8	0.59	0.21	2.20	3712	12.74
10	0.29	0.19	1.91	2464	5.51
15	1.10	0.38	2.35	6762	25.77
17	0.10	0.07	2.04	352	2.1
18	0.19	0.14	2.45*	1061	4.67
20	0.75	0.24	2.16	5229	16.04
21	2.73*	0.90*	2.14	18521*	58.66*
23	0.23	1.17	1.77	386	4.07
24	0.72	0.43	2.05	3859	14.59
25	0.35	0.20	2.35	2284	8.16
27	7.13*	1.60*	2.23	7562	152.86*
28	0.42	0.17	2.16	2362	9.51
29	0.24	0.07	2.20	926	5.27
30	2.30	0.66	1.88	4251	43.57*
31	0.71	0.32	1.81	5265	12.49
32	0.21	0.07	2.13	1106	4.37
33	0.02	0.01	1.74	197	0.41
34	0.37	0.09	2.35	2002	8.79
35	0.17	0.05	1.80	683	3.09
36	0.96	0.31	2.05	2278	21.88
38	2.41*	0.88*	1.94	2505	47.32*
40	0.31	0.23	2.23	2450	6.9
48	0.42	0.28	2.15	2513	9.05
49	0.38	0.25	2.22	3198	8.35
50	0.06	0.12	1.78	366	1.14
51	2.31	0.95*	1.92	4727	44.36
52	0.11	0.08	1.91	504	2.18
61	0.37	0.27	2.14	1569	7.98
63	0.18	0.11	1.96	856	3.55
64	0.13	0.18	1.80	478	2.2
65	3.14*	0.67	2.26	4784	65.14*
66	0.09	0.10	2.20	940	2.06
72	1.13	0.52	2.09	4719	21.86
73	0.19	0.04	2.14	787	3.94
74	0.71	0.32	1.99	5264	14.21
74	0.34	0.19	1.74	672	5.94
76	0.18	0.06	2.04	926	3.48
77	0.75	0.43	2.29	6504	17.35
78	0.83	0.25	2.61*	6094	21.38
79	0.35	0.20	2.24	1852	7.86
80	0.15	0.23	2.31	719	3.53
81	0.11	0.08	1.81	366	2.05
CV (%)	49.42	38.81	8.44	49.01	48.66

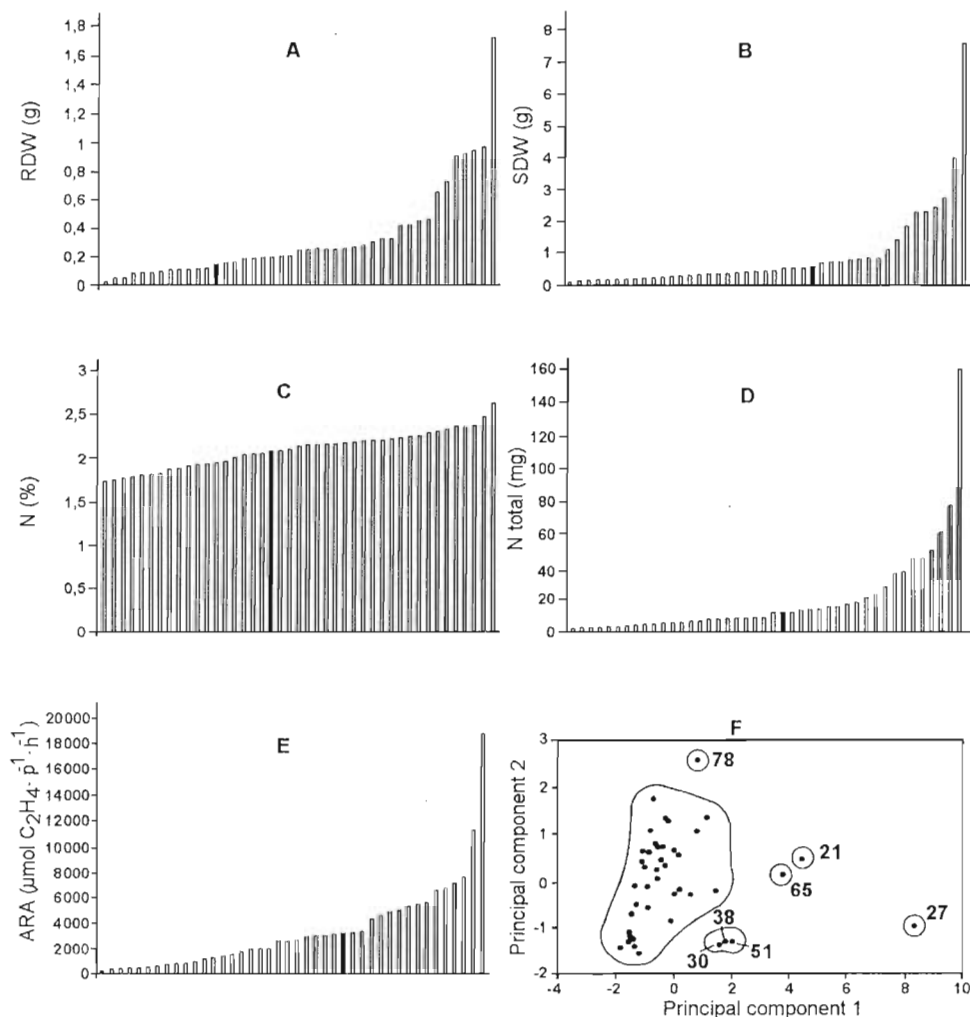


Figure 1 - Mean distribution patterns of *Stylosanthes scabra* regenerants and controls for the following traits root dry weight (RDW) (A), shoot dry weight (SDW) (B), shoot N content (N%) (C), Total N (D), acetylene reduction activity (ARA) (E), and scattergram for principal components 1 and 2 with clusters grouped by UPGMA method (F). Controls are indicated by solid bars.

variation was detected for all traits by comparing the regenerant means with those of the control.

Generally, the overall means of the regenerants were little altered compared to the control. However, some extreme somaclones could be identified with much larger values than the control. For example, the batch # 27 presented significant different values for the traits SDW, RDW and Total N (Table I).

Concerning the dispersion of data, a wide variability was detected among the regenerants for all traits that exhibited considerable range in their expression (Figure 1 A-E). Among the principal components, the first two explain more than 90% of the variation in the sample indicating that the six clusters were not arbitrarily formed. The regenerants # 21, 27 and # 65 were set apart by the multivariate analysis as shown in Figure 1F. The regenerants # 30, 51, and 38 formed a distinct group, concerning all variables. Altogether they represented 14% of the population.

Stylosanthes is a legume that is grown in areas of the tropical world, mainly Australia and Latin America, that are subject to recurring periods of drought stress and in poor and infertile soils. In this situation Stylo-Rhizobia symbiosis as a source of fixed nitrogen is always emphasized. Thus, biological N₂ fixation is a very important characteristic for providing the success for plant growth and establishment. Somaclonal variation should be a useful source of nodulation-related variants.

Since several plant loci are involved in nodulation (Dudley and Long, 1989) expressed by plant cell divisions and concomitant root hair curling it is possible that nodulation and N₂ fixation characteristics (such as nodule number and size, and ARA) were altered during *in vitro* phase explaining the variability observed in the present work. Variation in DNA methylation has been proposed as a mechanism that may elucidate the wide range of changes that can occur

after tissue-culture propagation, and may explain at least the changes described above (Kaepler and Phillips, 1993; Smulders et al., 1995).

Publication supported by FAPESP.

RESUMO

Plantas de *Stylosanthes scabra*, um poliplóide da família das leguminosas, tolerante à seca e adaptado a solos ácidos, foram regeneradas a partir da cultura de calos oriundos de folha. Um total de 168 regenerantes foram cultivados em vasos de Leonard em um experimento inteiramente casualizado. Fixação de N₂ e desenvolvimento vegetativo foram indiretamente avaliados por peso seco da parte aérea, peso seco da raiz, conteúdo de N e atividade de redução de acetileno. Os resultados mostraram maior variação nos regenerantes que nas plantas não submetidas à cultura de tecidos usadas como controles. Diferenças significativas foram encontradas para todos os caracteres associados à fixação de N₂.

REFERENCES

- Bussab, W.O., Andrade, D.F. and Myazaky, E.S.** (1990). Introdução à Análise de Agrupamentos. In: *Simpósio Nacional de Probabilidade e Estatística*, 9. IME/USP, São Paulo.
- Consoli, L., Vieira, M.L.C., Souza Jr., C.L. and Garcia, A.A.F.** (1996). Tissue culture effects on quantitative traits in *Stylosanthes guianensis*. *Braz. J. Genet.* 19: 469-474.
- Dudley, M.E. and Long, S.R.** (1989). A non-nodulating alfalfa mutant displays neither root hair curling nor early cell division in response to *Rhizobium meliloti*. *Plant Cell* 1: 65-72.
- Kaepler, S.M. and Phillips, R.L.** (1993). Tissue culture-induced DNA methylation variation in maize. *Proc. Nat. Acad. Sci. USA* 90: 8773-8776.
- Larkin, P.J. and Scowcroft, W.R.** (1981). Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60: 197-214.
- Mardia, K.V., Kent, J.T. and Bibby, J.M.** (1979). *Multivariate Analysis*. Academic Press, London.
- Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and biosays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Norris, D.O. and Date, R.A.** (1976). Legume Bacteriology. In: *Tropical Pasture Research. Principles and Methods*. C.A.B. (Commonw. Bur. Pastures Field Crops Hurley Berkshire. Bull.) 51: 134-174.
- Phillips, R.L., Kaepler, S.M. and Peschke, V.M.** (1990). Do we understand somaclonal variation? In: *VIIIth International Congress on Plant Tissue Cell Culture Proceedings*. Kluwer, Dordrecht, pp. 131-141.
- Smulders, M.J.M., Rus-Kortekaas, W. and Vosman, B.** (1995). Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. *Theor. Appl. Genet.* 91: 1257-1264.

(Received November 5, 1996)