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Somaclonal variation in rice after two successive cycles of mature embryo derived callus culture in the presence of NaCl

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Abstract

Two successive cycles of mature embryo-derived callus culture separated by one cycle of sexual reproduction of R_0 regenerated plants were performed using two rice (*Oryza sativa* L.) cultivars in order to gain information upon the nature of somaclonal variation in this species. Plants regenerated after one cycle of tissue culture exhibited higher variability and lower performances than those of initial cultivar. A second cycle performed using R_1 embryos as explants showed that the cellular component of salt resistance in terms of growth and regenerating abilities selected during the first cycle could be transmitted to the progenies. The extent and the nature of somaclonal variation depended on the identity of R_0 mother plant and culture conditions, somaclonal variation being strongly reduced in some families obtained from salt-treated calli.

Additional key words: genetic variation, *in vitro* selection, *Oryza sativa*, salinity, tissue culture.

Introduction

Somaclonal variation refers to variation arising in cell cultures, regenerated plants and their progenies (Larkin and Scowcroft 1981, Skirvin *et al.* 1994, Karp 1995). Its usefulness in plant breeding as a new source of variability is highly dependent on its nature since a high proportion of the reported somaclonal variation is of physiological or epigenetic nature and thus, could not be used in plant breeding programs (Valles *et al.* 1993, Vasil and Vasil 1994). Further information, however, is needed about its precise nature, especially in rice, since recent works demonstrated that tissue culture techniques could be successfully used in the improvement of this species for tolerance to salinity (Zhang *et al.* 1995, Winicov 1996, Lutts *et al.* 1999) or other abiotic stresses (Adkins *et al.* 1995, Bertin *et al.* 1996). In contrast, when transformation methods are used, somaclonal variation complicates the evaluation of the effects of introduced foreign gene and constitutes a major problem.

In *Oryza sativa*, mature embryo is the most widely used explant for obtaining calli. The aptitude to *in vitro* culture in terms of callus induction, growth and regenerating abilities as well as the sensitivity to somaclonal variation are highly dependent on the genotype (Peng and Hodges 1989, Adkins *et al.* 1995, Bertin *et al.* 1996, Lutts *et al.* 1998). Hence, in this homozygous diploid species, two successive cycles of *in vitro* culture separated by one cycle of self-pollination of regenerated plants could indirectly afford information on the nature of somaclonal variation. Indeed, if somaclonal variation is only of physiological nature, plants regenerated after one cycle of *in vitro* culture are genetically identical to those of initial cultivar. Then, if these self-pollinated regenerated plants are used as sources of explants (*i.e.* mature embryo) for a second cycle of *in vitro* culture in the same conditions, the extent and expression of somaclonal variation observed among plants regenerated after this second cycle should be the same as among plants obtained after the first cycle of *in vitro* culture. However, if this is not the case, one can hypothesize that plants regenerated after the first

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - benzyladenine; IAA - indole-3-acetic acid; IC - initial cultivar; IKP - I Kong Pao; K - relative potential somaclonal variation coefficient; LS - Linsmaier and Skoog; NAA - 1-naphthalene acetic acid; RGR - relative growth rate.

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cycle are not genetically similar to those of initial cultivar and that their progenies may therefore respond differently to *in vitro* culture.

Previous work also suggested that the expression of somaclonal variation could be influenced by the presence of stressing agents during the proliferation phase of *in vitro* culture (Lebrun *et al.* 1985, Rowland *et al.* 1988). In a recent study (Lutts *et al.* 1998), we demonstrated that NaCl added to proliferation medium mainly restricts the physiological component of somaclonal variation exhibited by regenerated rice plants. However, we do not have any information concerning the effects of NaCl on the putative genetic component of somaclonal variation and especially, on the genetic properties involved in subsequent response to a second cycle of *in vitro* culture and sensitivity to somaclonal variation.

The present work was undertaken to obtain additional information on the nature of somaclonal variation in rice by determining the influence of a first cycle of *in vitro* culture, in the presence or absence of NaCl, on variation expressed among plants issued from a second cycle of *in vitro* culture in the same conditions.

Materials and methods

Plant material and first cycle of *in vitro* culture: Seeds of rice (*Oryza sativa* L.) cultivars I Kong Pao (IKP, salt sensitive) and Aiwu (moderately resistant) were obtained from International Rice Research Institute, Philippines. Mature embryo-derived calli were obtained as previously described (Lutts *et al.* 1996, 1998) on a modified LS medium (Linsmaier and Skoog 1965) supplemented with 0.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg dm^{-3} 1-naphthalene acetic acid (NAA), 1 mg dm^{-3} benzyladenine (BA), 100 mg dm^{-3} myo-inositol, 0.1 mg dm^{-3} thiamine-HCl and 3 % (m/v) sucrose; pH was adjusted to 5.8 before adding 3 g dm^{-3} *Gelrite* (*Phytagel*; *Sigma Chemical Co.*, St. Louis, USA) and autoclaving for 20 min at 120 °C and 150 kPa. Three embryos were placed in each Petri dish and maintained in the dark at 25 °C. Six week-old calli were transferred to the same medium supplemented with 0, 200 or 300 mM NaCl (30 calli per treatment and per cultivar) and maintained for a further period of six months, with monthly estimation of fresh mass and transfer onto fresh medium. Relative growth rate of each callus was determined as

$$RGR = (\ln M_f - \ln M_i)/(\Delta t)$$

where M_i and M_f are initial and final fresh masses, respectively, and Δt the duration of exposure to salt (*i.e.* 30 d).

Plant regeneration from calli occurred in the absence of salt on half-strength LS salt medium (pH 5.8) supplemented with 1 mg dm^{-3} NAA, 1 mg dm^{-3} BA, 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine-HCl, 100 mg dm^{-3} tryptophan, 2 % (m/v) sucrose and 3 g dm^{-3} *Gelrite* in a growth chamber at 25 °C under a 12-h photoperiod (irradiance *ca.* 120 µmol(photon) m^{-2} s^{-1}). Calli presenting at least one developing shoot (height ≥ 1 cm) were then transferred for one month under the same environmental conditions as for plant regeneration in glass vessels (85 × 85 mm) containing 12 cm^3 of rooting medium (LS medium supplemented with 1 mg dm^{-3} IAA, 3 % (m/v) sucrose and 3 g dm^{-3} *Gelrite*). Regenerated R_0 plants were acclimatised in greenhouses, in polyethylene pots (150 × 150 mm) containing a mixture of loam and compost (1:1) under natural lighting supplemented by *Philips* fluorescent lamps (minimum 300 µmol(photon) m^{-2} s^{-1}; 12-h photoperiod) and maintained in the absence of stress. Pots were randomly rearranged in the greenhouse every 4 d. Night temperature was fixed at 25 ± 2 °C; day temperature fluctuated between 25 and 33 °C and relative humidity between 50 and 70 %. Survival percentages of acclimatised seedlings were recorded after 3 weeks considering as dead a seedling with all leaves necrosed. Mean number of viable R_0 plants produced per regenerating callus was then estimated for each treatment (cultivar × NaCl dose in proliferation medium). Total numbers of flowering and fertile plants were calculated according to Lutts *et al.* (1995) (Fig. 1).

Second cycle of *in vitro* culture and quantification of somaclonal variation: For each treatment, three fertile R_0 plants that produced at least 30 seeds and regenerated from distinct calli were randomly choosen. Mature embryos obtained from these seeds (termed R_1 families hereafter) were used as explants for a second cycle of *in vitro* culture. The calli thus obtained were transferred onto selecting medium containing the same NaCl dose as the one used for obtaining R_0 parental plant. For each treatment, 30 calli obtained from seeds of initial cultivar were used as control (one cycle of *in vitro* culture, termed C_0 hereafter). Culture conditions, modalities of plant regeneration and plant acclimatisation were the same as those used for the first cycle of *in vitro* culture. Plants obtained at the end of the second cycle of *in vitro* selection and termed C_1R_0 were maintained in the absence of stress until maturity. R_0 plants obtained from C_0 calli (one cycle of *in vitro* culture) constituted a first control and 30 plants of initial cultivar (directly issued from germinated seeds) were used as a second control. Both controls were also grown up to maturity in the absence of stress.

Data were recorded on each fertile plant: plant height, total number of tillers, percentage of fertile tillers, mean

panicle length, mean number of spikelets per panicle, spikelet fertility and mean mass of 100 grains were estimated according to Lutts *et al.* (1995). Sterile plants were not considered for the quantification of somaclonal variation. Somaclonal variation is quantified by a relative potential somaclonal variation coefficient (Lutts *et al.* 1998) defined as $K = (V_{RP} - V_{IC}) / V_{IC}$ where V_{RP} is the variance of the population of regenerated plants (R_0 or C_1R_0) and V_{IC} the variance exhibited by the initial cultivar maintained under the same experimental conditions. This coefficient was calculated for each of the above mentioned parameters and for populations of regenerated plants (R_0 or C_1R_0) obtained from each treatment.

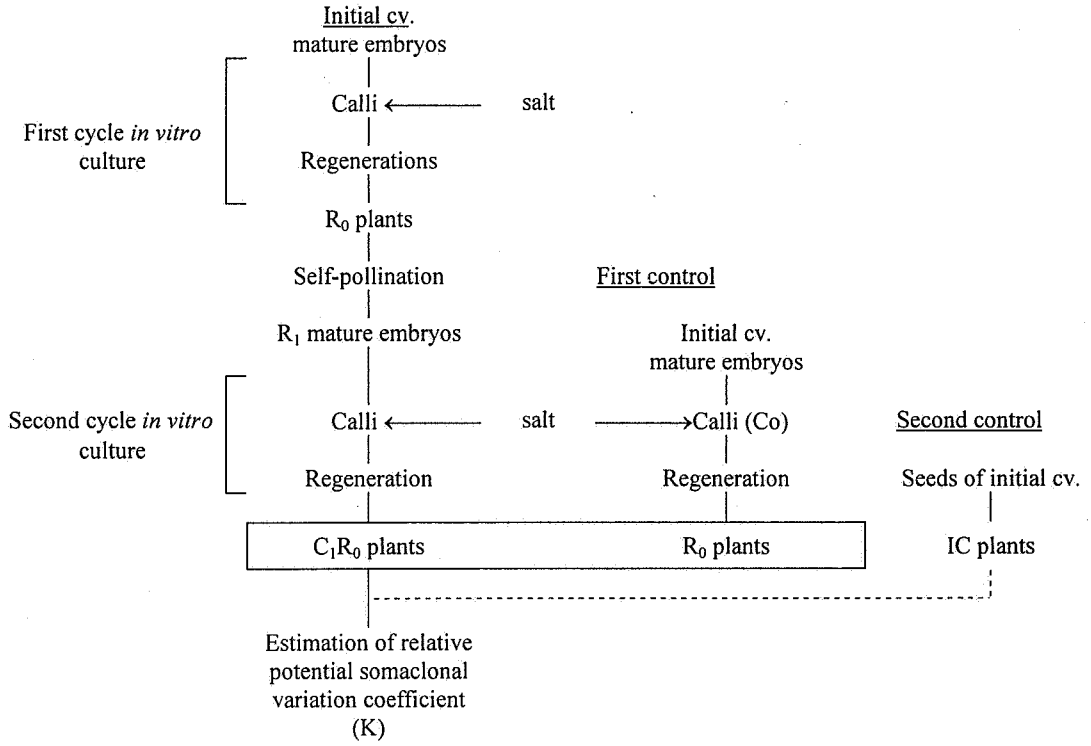

Fig. 1. General scheme of *in vitro* selection procedure. C_1R_0 plants were obtained after two successive cycles of mature embryo-derived callus culture separated by one cycle of sexual reproduction of regenerated plants. R_0 plants obtained after only one cycle of *in vitro* selection (C_0 calli) were used as a first control whereas plants of initial cultivar (IC) were used as a second control. For C_1R_0 and R_0 plants, a relative potential somaclonal variation coefficient was calculated (see text for details).

Statistical analysis: Relative growth rates (RGR) determined during proliferation phase were treated by a two-way analysis of variance (cultivar × NaCl dose). Number of viable R_0 or C_1R_0 plants produced per regenerating callus and total number of tillers were transformed according to Anscombe (1948) while percentages data such as fertile tillers and spikelet fertility were transformed according to Gabriel (1963). Means of regenerated plant populations (R_0 or C_1R_0) originated from each treatment were then compared to those of the corresponding initial cultivar by a Student *t*-test modified as recommended by Welch (1937) taking into account differences in variances and number of repetitions between treatments. The variance exhibited by each population of regenerated plants was compared to that of the initial cultivar by the *F*-test modified according to Box and Andersen (1955).

Results and discussion

Callus growth and regeneration: Calli of R_1 families obtained in the absence of salt stress presented slightly lower (IKP) or similar (Aiwu) mean RGR than calli obtained from bulked seeds of inital cultivar (Table 1). When the R_0 parental plant was regenerated from a salt-selected calli (families 4 to 9 and 13 to 18), however, mean RGR exhibited by calli obtained from R_1 seeds were clearly higher than those exhibited by calli obtained from initial cultivar and maintained in the same conditions. Moreover, mean RGR value was also higher, in these cases, than that of parental callus from which R_0 mother plant was obtained (Table 1). Thus, a first cycle of

in vitro selection improved the behaviour, in terms of growth, of calli obtained from progenies of regenerated plants. This suggest that a cellular component of salt resistance contributing to a better growth in the presence of NaCl have been selected during the first cycle of *in vitro* culture and transmitted to the progeny of regenerated plants. Previous works demonstrated that explants obtained from *in vitro* salt-selected R_0 plants gave rise to salt-resistant cell lines in *Poncirus trifoliata* and *Vigna radiata* (Beloualy and Bouharmont 1992, Gulati and Jaiwal 1993). In these studies, however, explants were directly obtained from leaf segments of R_0 regenerants without any cycle of sexual reproduction, while in the present case, scutellum cells proliferated to form a callus and the better behaviour during the second cycle of *in vitro* culture is therefore representative of salt resistance in R_1 generation. This point, and the fact that regenerated R_0 mother plants were maintained for a long

Table 1. Comparative performances of calli obtained from seeds of R_1 families and seeds of initial cultivar (control) in two rice (*Oryza sativa* L.) cultivars IKP (salt sensitive) and Aiwu (salt resistant). Mean relative growth rates of calli originated from R_1 embryos and of the parental calli from which R_0 mother plants were regenerated were estimated for a six months proliferation period. For each NaCl dose, data are means for three independent R_1 families. Differences between mean values of somaclonal families and mean values of the corresponding control are non-significant (ns) or significant at $P = 0.05$ (*) or $P = 0.01$ (**).

Cultivar	R_1 family number	NaCl [mM]	Number of calli	RGR [mg g^{-1} d^{-1}] R_1	RGR [mg g^{-1} d^{-1}] R_0	Regeneration [%]	Survival of plants [%]	Number of fertile plants
IKP	control	0	30	16.3	-	46.7	84.8	45
	1-3	0	84	13.9*	15.1	42.3^{ns}	85.8^{ns}	107
	control	200	30	9.8	-	43.3	67.8	26
	4-6	200	58	12.4**	9.4	74.3**	85.4*	86
	control	300	30	4.5	-	53.3	45.0	11
	7-9	300	68	7.4*	4.2	60.3*	72.9**	49
Aiwu	control	0	30	16.2	-	40.0	78.9	67
	10-12	0	62	15.2^{ns}	16.3	43.3^{ns}	76.2^{ns}	117
	control	200	30	12.8	-	30.0	63.1	17
	13-15	200	67	15.8**	12.6	60.1**	57.4^{ns}	78
	control	300	30	4.4	-	30.0	54.1	12
	16-18	300	53	6.5**	4.4	42.2*	85.1**	89

Table 2. Growth of plants of initial cultivar (control) and plants regenerated after one (R_0) or two (C_1R_0) cycles of *in vitro* culture in rice (*Oryza sativa* L.). Data are pooled for the three C_1R_0 families analysed in each treatment corresponding to a given dose of NaCl added to proliferation medium. For each population, the number of replicates correspond to the number of fertile plants given in Table 1. For a given cultivar values of the same column followed by the same letter are not significantly different at level $P = 0.05$.

Cultivar	NaCl [mM]	Plant generation	Plant height [cm]	Tiller number [plant^{-1}]	Fertile tillers [%]	Panicle length [cm]	Spikelet fertility [%]
IKP	-	control	60.3a	6.0a	61.4a	18.8a	73.2a
	0	R_0	57.8b	5.4b	61.8a	17.4ab	66.1b
	0	C_1R_0	60.1a	5.3b	59.6a	17.0b	69.6a
	200	R_0	55.2b	5.0b	56.4b	16.3c	60.3b
	200	C_1R_0	53.6b	4.9b	54.9b	15.9c	58.1bc
	300	R_0	53.8b	4.9b	48.2c	15.7c	50.4c
	300	C_1R_0	50.9c	4.8b	49.3c	16.0c	53.5c
Aiwu	-	control	64.8a	7.4a	70.1a	19.8a	70.2a
	0	R_0	58.3a	6.2b	64.6b	18.4a	69.8a
	0	C_1R_0	57.9a	7.0a	64.0b	19.0a	71.4a
	200	R_0	50.2b	5.8b	53.8c	16.8b	56.2b
	200	C_1R_0	52.1b	5.6bc	54.1c	17.2b	58.3b
	300	R_0	54.1b	5.2c	52.9c	17.0b	47.8c
	300	C_1R_0	50.5b	5.4c	50.1c	16.5b	47.4c

time in the absence of salt suggest that the improvement of the cellular component of salt resistance is of genetic nature and does not proceed from a simple physiological adaptation. The improvement of material previously selected in the presence of NaCl was also noticed as far as regeneration and survival percentages are considered (Table 1), although such improvement was more evident at 200 mM NaCl for the first parameter and 300 mM for the second one. In contrast, other parameters such as callogenesis percentages or mean number of plantlets obtained per regenerating callus were not significantly different for calli obtained from R_1 seeds on the one hand and those obtained from seeds of initial cultivar on the other hand (detailed data not shown).

Growth of regenerated plants: Growth characteristics and yield-related parameters exhibited by regenerated plants after one cycle of *in vitro* culture (R_0 plants) were lower than those of plants obtained from seeds of initial cultivar (Table 2). Such a negative effect of *in vitro* culture on subsequent performances of regenerated plants has already been reported by other authors (Lee *et al.* 1988, Kaepler and Phillips 1993). Individual components of yield are multigenic characters and their expression depends on complex metabolic interactions between numerous factors: it is thus not surprising that modifications induced by *in vitro* culture often lead to a decrease in agronomic performances. The presence of NaCl in culture medium slightly increased this negative influence of *in vitro* culture suggesting that conditions prevailing during cell proliferation could have a decisive influence on the behaviour of regenerated plants. An influence of genotype cannot be excluded since this deleterious effect of *in vitro* culture was not observed by Adkins *et al.* (1995) or Winicov (1996) working with other rice cultivars. It is also of special interest to notice that the deleterious effect of *in vitro* culture was not accentuated by a second phase of *in vitro* culture for most parameters exhibited by C_1R_0 plants (Table 2). The only noticeable exception concerned the mean number of spikelets per panicle which was strongly decreased in families No. 2, 3 (36.3 and 33.2, respectively, comparatively to 48.7 in R_0 issued from IKP) and 11 (41.2 comparatively to 62.3 in R_0 issued from Aiwu). Surprisingly, in contrast to the above-mentioned negative influence of NaCl, it is interesting to note that calli of these 3 families were maintained in the absence of stress.

Influence of NaCl added to *in vitro* culture medium on somaclonal variation: Relative potential somaclonal variation coefficient (K) was always positive for R_0 plants regenerated after one cycle of *in vitro* culture (Fig. 2) for five of the seven studied parameters. Thus, *in vitro* culture increased the variability of regenerated rice plants as already shown in other works (Adkins *et al.* 1995, Bertin *et al.* 1996, Lutts *et al.* 1998). The magnitude of this increase varied with the parameter considered and also depended on the NaCl dose added to proliferation medium: for example in the absence of salt stress, K values for fertile tillers percentages were most often higher than K values for spikelet fertility whereas an opposite trend was recorded for the material regenerated from salt-stressed calli. Previous studies already demonstrated that the extent of somaclonal variation varied according to parameters (Schaeffer *et al.* 1984, 1986, Griga *et al.* 1995). Up to now, there is no satisfactory explanation for such a phenomenon, due to our poor knowledge of the precise nature of somaclonal variation and to the genetic complexity of the analysed parameters. For a given parameter, K values also differed between cultivars (Fig. 2).

K values were lower when R_0 regenerated plants were obtained from salt-stressed calli comparatively to R_0 plants regenerated from non-stressed cell lines, except for spikelet fertility. This could be due to the fact that NaCl stress during proliferation phase induced the mortality of some of the modified cells while such cells were still able to proliferate and even regenerated in the absence of stress. If this is the only explanation, then R_0 plants obtained from salt-stressed calli originated mostly from non-modified cells and should therefore be roughly identical to plants of initial cultivar and perform almost similarly. Table 2 clearly shows that an opposite trend was recorded in the present experiment; other experiments are thus needed to explain why a decrease in variability is associated with a decrease in mean performance.

C_1R_0 plants obtained from non-stressed calli (families 1 to 3 and 10 to 12) presented comparable K values to R_0 plants. For some families previously selected in the presence of salt, however, a strong decrease in K values was found and negative values were even recorded in families No. 7, 8, 15 and 16, suggesting that this regenerated material had a more uniform behaviour than initial cultivar. It should be emphasized that in these families, the decrease in K values concerned all parameters simultaneously and not only a single parameter as would be expected for an erratic decrease in the variability of regenerated material. In contrast, other families (No. 5, 9, 17 and 18) still exhibited high K values. It seems therefore that the extent of somaclonal variation quantified at the end of a second cycle of *in vitro* culture depended, on the one hand, on the conditions prevailing during the first cycle of *in vitro* culture (*i.e.* the presence or absence of stress) and, on the other hand, on the identity of parental R_0 plants.

If somaclonal variation was the consequence of a simple physiological perturbation, as suggested by Vasil and Vasil (1994) to account for other results, K values should be the same, for a given NaCl dose, after the first

and the second cycle of *in vitro* culture. This, obviously, is not the case and suggest that somaclonal variation in rice does present a genetic "heritable" component, as previously suggested (Lutts *et al.* 1998, 1999). Another possible explanation would be that variation observed among regenerated plants resulted from a genetic heterogeneity of cultivated explants (Vasil 1986, Skirvin *et al.* 1994). However, this would suggest that scutellum cells were highly heterogenous in plants regenerated from non-stressed calli and rather uniform in some plants obtained from stressed ones, a phenomenon that seems

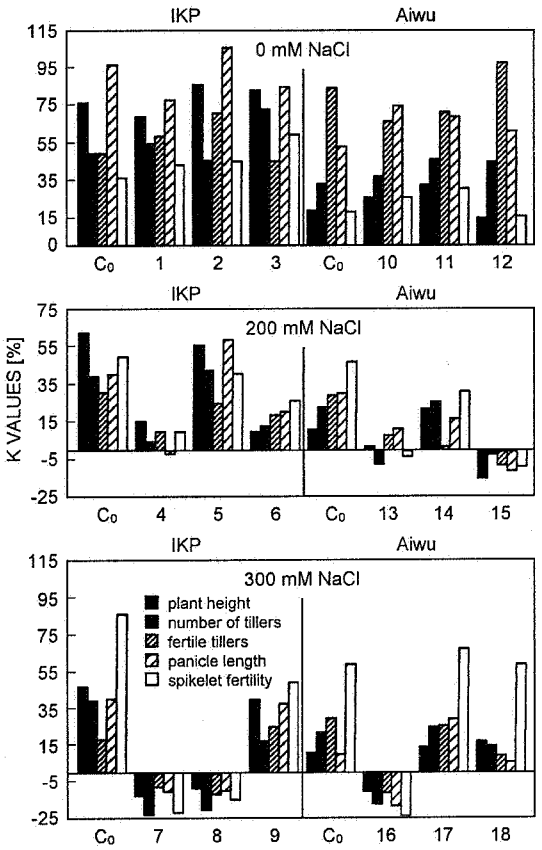

Fig. 2. Relative potential somaclonal variation coefficient, K [%] exhibited by R_0 plants regenerated after one cycle of in *vitro* culture and by C_1R_0 families regenerated from R_1 seeds-derived calli (1 to 18). Calli were maintained during six months in the presence of 0, 200 or 300 mM NaCl and regenerated plants were acclimated in the absence of salt. K values are given for plant height, total number of tillers, percentages of fertile tillers, panicle length and spikelet fertility.

hardly explainable. A third explanation would be that seeds of a same family used for the second cycle of *in vitro* culture were collected on one single R_0 plant and were therefore more uniform than seeds used for the first cycle of *in vitro* culture which were obtained from a bulked sample of seeds. This explanation remains unsatisfactory since families 1 to 3 and 9 to 12 were each the progeny of one single R_0 plant but still exhibited high K values after the second cycle of *in vitro* culture.

Finally it could be postulated that somaclonal variation resulted from genetic modifications occurring during proliferation phase but that NaCl indirectly influenced the molecular events leading to somaclonal variation such as DNA methylation (Sabbah *et al.* 1995) or the production of mutagenic free oxygen radical species (Becaña *et al.* 1998). Since sensitivity to somaclonal variation is highly dependent on genotype (Karp 1995, Adkins *et al.* 1995, Bertin *et al.* 1996, Lutts *et al.* 1998), it is conceivable that genetic modification occurring during a first cycle of *in vitro* culture and transmitted to progenies after sexual reproduction could influence the sensitivity to somaclonal variation of material cultivated during a second cycle of tissue culture. On another hand, it cannot be excluded that K values calculated after the second cycle of *in vitro* culture may overestimate somaclonal variation since a significant part of variability could be explained by segregation during sexual reproduction in R_0 of heterozygous genes resulting from point mutation during the first cycle of *in vitro* culture. This, however, would imply that R_0 plants obtained from unstressed calli exhibited a higher level of heterozygozity than those obtained from NaCl-treated calli.

Amzallag *et al.* (1995) reported that in *Sorghum bicolor*, a specific phase of increase in variability occurred between day 6 and 16 following exposure to salinity. Such an increase was specifically related to the adaptation process. Although it occurred at the early vegetative phase of development, it may also influence the expression of late reproductive characters as a result of non-oriented changes in genome expression which were stable and transmissible (Amzallag 1996). In our work, salinity was applied only during phases of *in vitro* culture but never at the whole plant level. Thus, variability reported here should be a result of somaclonal variation *sensu-stricto*, even if the presence of NaCl in the medium may have an impact on underlying mechanisms leading to somaclonal variation.

The present study reinforces the hypothesis that somaclonal variation in rice does involve a genetic component and that NaCl added to proliferation medium could have an impact on the genetic properties conditioning the sensitivity to somaclonal variation occurring during a second cycle of *in vitro* culture. Salt-resistant variants were recently identified among tissue culture derived plants and were physiologically characterized (Lutts *et al.* 1999): this promising material is now used to determine the genetic modifications associated with an improvement of salinity resistance.

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