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Salt tolerance through increased vigor in a flax line (STS-ll) selected for salt tolerance in vitro

A. McHughen

Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

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Summary. Progeny of a flax (*Linum usitatissimum* L. cv "McGregor") plant, regenerated from a cell line selected in vitro for salt tolerance (designated STS-ll) was tested for its performance over two generations in normal and in saline soil against its parent variety. Germination, seedling height, flowering, seed set and seed yield in controlled greenhouse conditions were recorded. The putative salt tolerant line was superior in saline soil for all parameters measured, indicating that the mechanism selected in cells in vitro was also active in whole plants, and that the trait is genetically stable and seed transmitted. Unexpectedly, the STS-ll line was also superior in the normal, non-stressed soil, indicating that the selected trait is not limited to salt tolerance specifically, suggesting a more general mechanism, such as a general increase in vigor.

Key words: *Linum usitatissimum* – Flax – Salt-tolerance – Stability – In vitro selection

Introduction

Excess salts in soil are a major agronomic problem in many parts of the world, and traditional breeding methods have met with limited success when addressing them. In recent years, several groups have tried to overcome the limitations of traditional breeding by selecting for salt tolerance in cell cultures. If the technique of cellular selection is to be usefully applied in crop improvement programs, the efficacy of the selection program, in terms of progeny performance in soil, will have to be demonstrated. Two critical questions need to be addressed. (1) Is the trait, selected at the cellular level, active at the whole plant level? (2) Is the trait genetically stable? It is interesting to note that in a recent review, Raghava Ram and Nabors (1985) cite 37 cases of cellular selection in vitro for salt tolerance, but in only six were plantlets regenerated. Putative salt tolerant cell lines of crop plants include, for example, tobacco (Dix and Street 1975; Nabors et al. 1975), alfalfa (Croughan et al. 1978), flax (McHughen and Swartz 1984) and chickpea (Pandey and Ganapathy 1984), but often plants were not regenerated from the selected cell lines. Where they were regenerated, the progeny typically were not analysed for salt tolerance, with some notable exceptions. Bhaskaran et al. (1986) used a hydroponics system to test progeny of a sorghum plant regenerated from a salt tolerant cell line in vitro. Spiegel-Roy and Ben-Hayyim (1985) tested their putative salt tolerant citrus plantlets in culture medium and Nabors et al. (1980) tested their salt tolerant tobacco progeny by watering them with a saline solution. In this report, the performance of progeny of flax plants regenerated from a salt tolerant cell line in saline-stressed and normal soil is presented. The results indicate that cellular selection can be a useful adjunct to traditional breeding programs.

Materials and methods

The presumptive salt tolerant cell line and subsequent regenerant were produced as described earlier (McHughen and Swartz 1984). Briefly, callus was initiated from hypocotyl segments of flax (*Linum usitatissimum* L. cv "McGregor"), macerated, and plated onto Murashige and Skoog (1962) medium containing 2 mg/l 2,4-D, with 2.5% sucrose and 0.8% agar. After several weeks of callus growth, the calli were chopped into small pieces (~ 1 mm^3) and transferred to fresh medium, this time containing no hormones but instead an excess of salts in the proportions and concentrations as found in a sample of local, highly saline soil. The total concentration was 2.5%–3%. After

Table 1. Germination (38 days after sowing) and seedling vigor (as determined by height of plants (in cm) 70 days after sowing) of McGregor and STS 11 seeds in normal and in saline soils*

	No. seeds sown	No. germinated	% germinated	No. plants at 70 days	Height (cm) 70 days after sowing ($\bar{x} \pm SE$)
Normal soil					
McGregor	48	34	70	34	24.4±3.6
STS 11 A	24	20	83	20	35.2±6.6***
STS 11 B	24	20	83	20	34.3±6.6***
Moderately/highly saline					
McGregor	48	24	50	26	16.7±3.5
STS 11 A	24	14	58	13	26.3±5.6***
STS 11 B	24	13	54	14	20.8±5.6*
Very highly saline					
McGregor	48	13	27	16	11.4±2.7
STS 11 A	24	13	54*	12	18.7±4.4***
STS 11 B	24	11	46*	10	16.1±4.7*

* Significant differences between McGregor and STS 11 within a soil type are indicated (*, *** = $P < 0.05$, 0.001, respectively). There were no significant differences between STS 11 A and STS 11 B

32 days on this medium, only a few discrete green spots remained alive amid necrotic cells. The green spots were excised and transferred to MS medium with 2 mg/l IBA, 1 mg/l kinetin and 3% sucrose. Shoots arose and were transferred to Gamborg et al.'s B5 medium (1968) to promote rooting (Murray et al. 1977). Plantlets were established in vermiculite and finally transferred to ordinary greenhouse potting soil to mature and set seed. Over 50 putative salt tolerant flax lines have been produced using this or slightly modified methods; one of these lines, the most advanced, is documented here.

Like most primary regenerants, the plant, called STS-ll (STS-l later proved salt tolerant but yielded poorly under non-stressed soil conditions), produced few seeds, so reliable first generation evaluations were precluded. However, the seeds were divided into two lots. One was sown into the standard greenhouse potting soil in 15 cm pots, one seed per pot. The electrical conductivity of this soil was 1.03 mS/cm. Into the same pots was sown a seed of the parent genotype, McGregor, taken from a batch of certified seed. The second lot of seed from the regenerant was sown into the same standard potting soil, but to which was added sufficient $MgSO_4$, $NaSO_4$, NaCl, and $CaCl_2$ to bring the conductivity to approx. 8 mS/cm as determined by sampling the soil as it was being prepared. The artificially saline soil, considered moderately saline, was distributed to 15 cm pots into which one seed of the progeny of the salt tolerant regenerant and one seed of the parent McGregor from a batch of certified seed were sown. In all, 20 pots of each saline and normal soil were prepared. Levels of salinity were measured by the 1 : 1 saturated paste method and determined by electrical conductivity both before seeding and after harvest to ascertain dramatic changes in salinity due to, e.g., leaching of salts through overwatering. Moderate saline stress is indicated by retarded growth, delayed development and reduced yield compared with non-stressed plants. Severe stress can result in non-germination or stunded seedlings followed by death prior to maturation.

The first experiment, testing the progeny of the regenerated salt tolerant plant against McGregor in normal and in moderately saline soil, suffered from poor germination of both genotypes in both soils, so the data collected were not as reliable as expected. Only 5 of 20 pots of normal soil had plants of both McGregor and STS-ll, and there were no significant differences between them for any measured parameter. Only 7 of 20 pots of saline soil in this experiment had both the control McGregor and the experimental STS-ll plants; of these, the two plants performed equally well (or poorly) in two pots for the parameters measured, while the STS-ll plant was visibly superior in the other five pots. Over the total population, the STS-ll was significantly superior in every parameter except 1,000 seed weight.

For the next generation test, two plants of STS-ll which performed well in saline soil were selected and designated A and B. This time, each pot was sown with two seeds of the parent genotype and one each from STS-ll A and B, for a total of four seeds per pot. Also in this experiment, new batches of artificially saline soil were made up to be moderately/highly saline (~ 12 mS/cm) and very highly saline (~ 15 mS/cm), in addition to the normal soil, for a total of three soil types tested. Observations were recorded for germination, height (to determine vigor), time to flowering, number of bolls, number of seeds, and total weight of seeds per plant.

Results

Third generation test

Germination. Here, 24 pots containing non saline (normal) soil, 24 with moderately/highly saline soil and 24 with very highly saline soil were sown with one seed each of the two STS lines (A and B) and two seeds of the control McGregor. Table 1 shows germination data from this test, scored 38 days after sowing. According to Fisher's exact test, STS-ll A and B are not significantly different from each other in germination in normal or in saline soils, and not different from the controls in nor-

Fig. 1. Condition of STS 11 and McGregor plants in saline soil. STS 11 B, control McGregor and STS 11 A plants (*left to right*) in very highly saline soil (electrical conductivity ~ 15 mS/cm). Note both STS 11 plants are of similar height and are flowering (STS 11 A has buds, STS 11 B is at anthesis), while the McGregor plant main axis has died. The production of axillary shoots by non-tolerant plants is a typical but futile response to severe saline stress

mal soils, but are significantly different from the control in the very highly saline soil ($P < 0.05$).

Late germination of seeds and "seedling death" account for differences in plant number between Table 1 (germination, seedling height) and other tables.

Intermediate height. Plant height was measured 70 days after seeding and the data presented in Table 1. In normal soil, both putative salt tolerant lines A and B were significantly taller than the McGregor ($P < 0.001$ for both), indicating that the lines derived from the salt tolerant cell line are more vigorous than their ultimate progenitor. Under saline soil stress, significance remains ($P < 0.001$) between the STS-ll lines and the control (Fig. 1). At this time of development, flower buds had just initiated on 6 of 89 plants from the STS-ll A and B lines, while none had initiated on any of the 76 control plants over all soil types. Eight of the 16 control plants in the very highly saline soil were severely wilted, while none of the STS-ll plants were so scored.

Time of flowering. At 32 days after intermediate heights were recorded, plants were scored for stage of flowering (Table 2) as "not flowering", "before anthesis" (early flowering), "recent anthesis", or "late flowering" – a plant with buds beyond anthesis and some well-formed bolls. This table also shows one of the effects of salt stress on plant development, that of retardation. All three lines show most flowering in the least saline soil, and the least flowering in the very highly saline soil. This diminished rate of development is not dissimilar to the effect of increasing salinity on germination; that is, as salinity increases, germination decreases.

By comparing flowering and not flowering in each soil type and across genotypes, lines A and B, either separately or combined, flower significantly earlier than McGregor ($P < 0.01$) and in no case is A significantly different from B, according to Fisher's exact test. Also, in every pot containing at least one control plant and one STS-ll plant, the STS-ll plant was first to flower. So, both as individuals in a pot and as a population across pots, STS-ll is first to floral transformation. This is true in both saline and non-saline soils. While the increase in salinity does retard flowering even in the STS-ll lines, only in the highest salinity level does the retardation in the STS-ll make it later to flower than the McGregor under non-stressed soil conditions.

Number of bolls. Flax is typically self-pollinated and highly self-fertile; the degree of outcrossing in the field is insignificant. Each fertile flower produces a boll when pollinated successfully and eggs fertilized, so the number of bolls is related to the number of fertile flowers. Table 3 shows the number of bolls produced per plant of STS-ll A and B and the control McGregor in saline and non-saline soils. STS-ll A is not significantly different from B in any of the soils tested, but STS-ll A and B each produced significantly more bolls than the control in each soil type ($P < 0.001$ for normal and moderately/highly saline soil, $P < 0.01$ for very highly saline soil). The McGregor control plants grown on very highly saline soil produced no bolls, and only six of the 25 control plants grown on moderately saline soil produced at least one boll. Of pots containing at least one STS-ll plant and one control plant, of which there were

Table 2. Flowering data for McGregor and STS 11 A and B in normal and saline soils. Precent-1: percentage flowering of plants present; Percent-2: percentage of plants flowering of seeds sown

Genotype Salinity level (mS/cm)	McGregor			STS 11 A			STS 11 B		
	0	12	15	0	12	15	0	12	15
No. plants	33	26	14	20	14	13	19	14	10
Not flowering	13	26	14	0	0	6	0	2	3
Before anthesis	7	0	0	0	2	3	0	3	5
Anthesis	10	0	0	1	6	4	1	7	2
Late flowering	3	0	0	19	6	0	18	2	0
Total flowering	20	0	0	20	14	7	19	12	7
Percent-1	60.6	0	0	100	100	54	100	85.7	70
Percent-2	41.6	0	0	83	58	29	79	50	29

Table 3. Number of bolls, seeds and seed weight (mg) per plant of McGregor and STS 11 A and B grown in saline and non-saline soils*

	Normal soil		Moderately/highly saline		Very highly saline	
	(*n*)	$\bar{x} \pm SE$	(*n*)	$\bar{x} \pm SE$	(*n*)	$\bar{x} \pm SE$
Bolls						
McGregor	33	2.5±2.5	25	0.7±1.4	14	0
STS 11 A	20	5.7±2.1***	14	8.9±3.9***	14	1.5±2.3**
STS 11 B	19	5.3±2.1***	15	6.1±3.9***	12	0.8±1.6**
Seeds						
McGregor	33	19.5±11.6	25	2.8± 5.4	14	0
STS 11 A	20	45.4±17.2***	14	74.5±33.6***	14	6.7± 9.4*
STS 11 B	19	42.9±16.4***	15	49.5±32.1***	12	8.5±10.6*
Seed weight						
McGregor	33	88.6±58.5	25	7.7± 18.8	14	0
STS 11 A	20	207.7±70.5***	14	305.3±149***	14	40.6±54.1*
STS 11 B	19	192.0±82.3***	15	202.6±136***	12	32.4±41.1*

* Significant differences between McGregor and STS 11 in each soil type are indicated (*, **, *** = $P < 0.05$, 0.01 and 0.001, respectively)

21 of normal soil, the control plant had more bolls in 2 pots and the STS-ll plant had more bolls in the other 19 pots. There were 18 such pots of moderately/highly saline soil; none of these contained more bolls than the STS-ll counterpart(s). Of nine pots of very highly saline soil containing plants from both genotypes, five had no bolls on any plant while the remainder had bolls only on the STS-ll plants.

Number of seeds. Normal flax pistils are pentacarpellate; each carpel has two locules, each with an ovule. Under ideal conditions, each boll can therefore produce 10 seeds. Typically, however, in the field, 7 or 8 seeds are produced per boll, with wide variation among bolls on a given plant. In the present experiment, bolls were harvested from each plant, threshed individually and the seed hand counted. The results are summarized in Table 3. Again, there was no significant difference between STS-ll A and B for number of seeds per plant within any soil type, but in both saline and non-saline soils there were significant differences noted between STS-ll A and B, either individually or together, and the controls ($P < 0.001$); the control yielded no seeds on the highly saline soil.

Anomalously, STS-ll A produced more seeds per plant in moderately/highly saline soil than it did in normal soil ($P < 0.01$). This is not exclusively due to fewer plants producing a similar number of seeds in the saline soil, because the mean number of seeds produced per seed planted (n = 24 for each of the STS-ll lines, 48 for the control, in each soil type) indicates higher productivity for STS-ll A in moderately/highly saline soil. STS-ll A produced a mean of 37.8 seeds per seed planted in normal soil, and 43.4 seeds per seed planted in moderately/highly saline soil. STS-ll B produced 33.9

seeds per seed planted in the normal soil and 30.9 seeds in the moderately/highly saline soil, while the McGregor produced only 13.6 seeds per seed sown in normal soil and 1.4 seeds in moderately/highly saline soil. This increase in productivity by STS-ll A in a higher salt stress is the only record of divergence between STS-ll A and B.

Total weight of seed per plant. The yield of a grain plant is determined by the total weight of seeds produced. Table 3 summarizes the data on the total seed weight per plant of STS-ll A and B and of the control McGregor in normal, moderately/highly saline and very highly saline soils. In individual pots, where at least one STS plant and one control plant were present, the combined yield of the control plants was superior to the combined yield of the STS-ll A and B plants in three of 21 pots of normal soil, and in none of 17 of those with moderately/highly saline soil. As there were no seeds produced on control plants in the very highly saline soil, pot to pot comparisons cannot be made.

Discussion

The nature of salt tolerance is poorly understood, primarily because the nature of salt stress is poorly understood. It is clear, however, that salinity stress has at least two components; one component is that of an osmotic stress from the increased water potential from saline ions present in abundance in the soil; the other is that of ion toxicity from overabundance of particular types of ions. A plant can be salt tolerant due to one or more of several possible mechanisms. These can range from fairly specific, simple genetic means such as chloride excluder genes (Abel 1969) or increased levels of certain substances though to protect cells from osmotic damage such as glycine betaine (Storey and Wyn-Jones 1975) or proline (Stewart and Lee 1974), to more complex genetic systems involving morphological mechanisms such as salt glands. Other mechanisms of salt tolerance include those bearing no direct relationship to salt metabolism. This includes those in plants which grow only in the wet season, thus avoiding the salinity stress imposed in the same soil during the dry season, or plants which have an increased vigor and are thus better able to withstand any kind of stress imposed, at least for short periods (recent reviews: Raghava Ram and Nabors (1985); Stavarek and Rains (1983); Greenway and Munns (1980)).

Cellular selection schemes are thought to provide the possibility of identifying certain agronomically valuable traits, such as some forms of salt tolerance, which are active at the cellular level. The flax line derived from a cell colony surviving a normally lethal saline culture analyzed in saline soil after one and two seed generations does show superiority over its parent line. The means by which this line is salt tolerant is not known, but the superiority over its parent is obvious in normal and saline soil, indicating that a non-specific mechanism of tolerance has been activated in cell culture. If this turns out to be true, then cellular selection might be applicable to other traits regulated by general mechanisms, instead of being directed exclusively toward those traits with a simple, specific nature such as herbicide resistance, disease resistance or specific metabolite production.

The results of the germination test indicate that the STS-ll line is more vigorous in every soil, but the differential is most pronounced in the most saline soil, where almost twice as many STS-ll seeds germinated as the McGregor (50% vs 27%). After 70 days of growth, this difference in vigor is more noticeable in the height of the plants. Under non-stressed soil condition, the McGregor plants average about 24.5 cm in height, while the STS-ll plants are about 34.5 cm, or approx. 50% taller on average than the controls. In moderately/highly saline soil, the height of the McGregor plants has reached only 16.7 cm, while the STS-ll plants, also retarded by the salinity, reached only approx. 23.5 cm (similar to the McGregor plants under non-stressed soil conditions); the differential, however, between the controls and the STS-ll remains approx. 50%. Under the highest level of salinity, the McGregor plants average only 11.4 cm, while the STS-ll plants have grown only to about 17 cm, again showing about a 50% differential over the control plants under a similar level of saline stress. The relative performance of McGregor and STS-ll plants remains the same, and so we presume that the two lines respond to salinity in the same way; that is, an increase in saline stress has a similar depressing effect, or regression, on both lines. This indicates that the reason for superiority of the STS-2 line over McGregor, and its survival as a cell line in lethally saline culture medium, is not due to any specific salt metabolism gene, but rather more likely due to a general increase in vigor. The flowering data also support this interpretation; a specific gene conferring salt tolerance alone would not contribute to significantly earlier flowering under non-saline soil conditions, but a "vigor" gene might. Interestingly, in one of the few other reports dealing with performance of progeny of salt tolerant selected cell lines, Bhaskaran et al. (1986) noted that in a hydroponics system testing their sorghum derived from a salt tolerant cell line, an increase in saline stress resulted in a decrease in productivity in both control and experimental lines, but that the selected line appeared more vigorous than the parent under non-stressed conditions. Although the differences were not statistically significant in any one parameter, the putative salt

tolerant line was superior to the parent in each of the six parameters measured. Perhaps they, too, identified a line salt tolerant due to increased vigor.

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