EXECUTIVE SUMMARY

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Excess soil salinity is a major factor limiting turfgrass growth, therefore, selection of suitable salt-tolerant (ST) genotypes is a high research priority. Although cell culture and biotechnology are potentially powerful tools for isolating superior, more tolerant, and unique selections, the ST traits must be effectively demonstrated at the whole plant level. The goal of this project was to develop a system to screen turfgrasses at both the cellular (callus) and whole plant level to verify ST traits, in order to more efficiently evaluate novel germplasm for breeders.

A stable, uniform whole plant microculture (WPMC) testing system was implemented to bridge in vitro laboratory research results and whole plant-level performance. WPMC offers a useful method for prescreening the vegetative stability of the cell-culture selected, putative salt tolerant turfgrasses, prior to producing R_1 seeds. Efficient methods for callus induction, proliferation, and plant regeneration were developed for several cultivars of St. Augustinegrass, bermudagrass, creeping bentgrass, and zoysiagrass. Cell-level screening for salinity was accomplished by introducing callus to a saline medium (0 - 1.5% Na_2SO_4) just prior to the regeneration of new shoots. Most of the regenerative capacity of the callus was lost during culture on saline medium. Only a small proportion of the salt-challenged callus lines were capable of producing regenerated turfgrass plants in spite of the salinity stress.

The regenerated plants were clonally propagated (shoot culture), and introduced to saline conditions in WPMC, in order to evaluate the putative ST selections for vegetative performance under stress. These whole plant level tests confirmed that salt tolerance was stable for only a fraction of the putative ST lines selected in cell culture. However, the cell screening did produce, in low frequency, turfgrass plants with significantly more ST overall than control (non-selected) plants. Current investigations are aimed adapting cells in liquid culture to gradually increasing levels of salt stress, to ensure that cells are uniformly exposed to salt and to increase the frequency of stable mutations.

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Summary of major project accomplishments, 1992

A Realistic Whole Plant Microculture Selection System For Turfgrasses

[Cell level selection for salt tolerance and confirmation of whole plant salt tolerance characteristics]

Development of complete cell culture and regeneration systems for selected turfgrasses.

In order to develop general techniques for manipulating a broad range of turfgrasses in vitro, the effects of explant type and developmental stage, plant growth regulators, carbon source, and cultural method were investigated for several cultivars of St. Augustinegrass, zoysiagrass, bermudagrass, and bentgrass.

Explants: unemerged inflorescences most consistently yielded friable callus with high potential for somatic embryogenesis. Nodes, seeds, or emerged (more mature) inflorescences produced callus, but frequently the tissue was not responsive to manipulation in culture. Seed explants were, however, suitable for zoysiagrass and bentgrass.

Plant growth regulators: 2,4-D was the only auxin which routinely produced callus with high regeneration capacity.

Carbon source: elevated sucrose levels (i.e 60 g l^{-1}) enhanced callus growth rate and friability.

Cultural method: agar-solidified culture medium, as opposed to liquid culture or liquid filter-paper bridges, supported best callus proliferation growth.

As a result of these investigations, a generalizable protocol for cell culture of several species of turfgrasses was developed, including the first reported case of efficient plant regeneration from St. Augustinegrass callus.

Selection of salt tolerant cell lines and follow-up evaluation of whole plant response under salt stress.

Callus samples from each of the turfgrasses were introduced to regeneration medium supplemented with 0, 0.5%, 1.0%, or 1.5% Na_2SO_4. The technique was a single-step selection method, since the cells experienced saline conditions only once prior to plant regeneration. This technique very rapidly permitted isolation of potentially ST lines from in vitro culture. Only a small fraction of these callus masses were capable of surviving and regenerating plants under the saline conditions. Well-rooted, regenerated plants were clonally-propagated either in a greenhouse, or in microculture, in order to generate sufficient numbers for screening whole plant performance. The clonal plants were screened in WPMC medium supplemented with the same salt levels (0-1.5% Na_2SO_4) used at the cell culture stage of the system. During this whole plant in vitro screening, the media was solidified with gelrite (rather than agar) in order to maximize visibility in the root zone. The stability of ST traits was evaluated by testing the root number, root area, and shoot area of WPMC plants under salinity stress and in the absence of stress. Machine vision was used to quantify these whole plant growth criteria.

In the final evaluations, callus and regenerated plants which were selected on 0.5% Na_2SO_4 regeneration medium produced whole plants that remained stable under the same salt concentrations. These ST selections, produced at very low frequency, performed significantly better than control (non-selected) plants on saline-supplemented growing medium. However, plants originally regenerated at higher salt concentrations (1.0 - 1.5 % Na_2SO_4) failed to demonstrate consistent ST characteristics at the whole plant level. This result suggests that salt adaptation mechanisms, rather than stable mutations under salt stress, were the mechanisms which permitted some callus masses to regenerate at high salt levels. Only gradual, low level salinity challenges in the cell culture resulted in production of entire plants with superior ST traits. These selections are currently growing under greenhouse conditions, to determine stand characteristics and to evaluate seed production.

In general, the single-step method of exposing turf callus to salt stress prior to regeneration does not lead to a high frequency of stable mutations in WPMC screening. There were apparent problems with reversion, indicating that the ST was an unstable trait in some cases. As intended, the WPMC prescreen effectively purged these unstable selections from time-consuming and costly subsequent tests in the greenhouse or field. Current tests (as described further below) now apply the salt stress to the cells in continuous liquid suspension culture over several subculture cycles. Although this strategy effectively reduces the regenerative capacity of the callus lines (dramatically), it is expected to produce a greater frequency of stable regenerates in whole plant tests.

Accomplishments under USGA sponsorship of this project:

Research under this project has involved 2 M.Sc. level graduate research assistants, 1 technical assistant, and two Jonathan Baldwin Turner College of Agriculture sponsored scholarship projects. The accomplishments included

a. Validation of the WPMC system. These studies were conducted to quantify whole turfgrass plant responses to salt in solution culture and whole plant microculture test environments, and to evaluate the latter [WPMC] as an alternative system for identifying salt resistance characteristics. Paired cultivars of bermudagrass (Cynodon spp. (L.) Pers.), creeping bentgrass (Agrostis palustris Huds) and St. Augustinegrass (Stenotaphrum secundatum (Walt) Kuntze) were tested in treatments ranging from control (no supplemental salt) to 32.4 dS m^{-1} conductivity (NaCl in the growth medium). Machine vision was used to evaluate growth and developmental changes in both environments, and permit non-destructive interim evaluations in WPMC. The research involved thorough characterization of morphometric (spatial) and photometric (spectral) responses of selected salt tolerant and salt sensitive turfgrass lines to root zone stress, and use of video image analysis to automatically quantify and validate each of these responses, which permitted use of larger sample sizes and more thorough screening in the analysis.

These results demonstrate that turfgrass plant responses to salinity can be non-intrusively monitored over the course of a screening test, in a simple, small-scale, low maintenance, highly repeatable microculture environment. We established strong linear regressions for shoot and osmotic adjustment parameters under increasing salinity levels in both solution culture and whole plant microculture. Analysis of covariance established that the slopes of regression lines for osmotic adjustment were the same in both environments, which indicates that whole plant microculture is a valid means of examining turfgrass responses under saline conditions. Root growth parameters (root length and area) were more variable in both solution and microculture. Whole plant microculture conferred additional advantages as a highly-controlled test system in terms of scale, timing, maintenance, and repeatability.

b. Development of cell culture/regeneration protocols for selected turfgrasses in order to test the whole plant microculture system as a bridge between biotechnology (manipulation of germplasm at the cell level and regeneration from cell culture) and field testing for enhanced stress tolerance. Overall objectives for this phase of the project were 1). to optimize a complete microculture system, including continuous callus culture, regeneration, adaptation of stable, uniform

whole plant cultures, and finally acclimation of whole plants to greenhouse and field settings, 2). to obtain stable salt tolerant [ST] cell lines after in vitro selection, which can be regenerated and subsequently tested at the WPMC level to verify ST, and 3). to identify the relative efficiency of cell level and whole plant level screening tests for ST, and establish the effectiveness of a WPMC system as a valid, efficient means for prescreening regenerated, putative ST lines from cell culture.

Efficient regeneration strategies were first established for zoysiagrass, St. Augustinegrass 'Seaside', common bermudagrass, and creeping bentgrass. A range of explants (seed, mature and immature inflorescences, immature embryos, and vegetative nodes) were investigated for callus production and regeneration experiments. In general, only immature inflorescences provided a reliable source of callus which could be maintained for at least 3 subculture rounds, and from which complete plants could be transferred and acclimated to the greenhouse. In order to develop working methodologies, both agar and liquid (bridge) culture systems were investigated, with a range of physical microenvironmental parameters (light regimes, temperatures) and chemical factors (hormonal). The WPMC evaluation system described here may permit promising new selections from in vitro research to be identified, screened, and rated prior to plant acclimatization and scale-up for field trials.

Finally, seed from the regenerated, putative ST turfgrass plants has been produced in the greenhouse, and progeny is under evaluation. In addition, all of the tests in the cell culture regeneration/ST screening/WPMC evaluation sequence are being repeated to verify the stability of the results.

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Current investigations/New directions:

Putative salt tolerant plants from the cell-level-to-whole-plant screening trials described above are currently being evaluated in the greenhouse, in order to examine genetic stability over a number of generations. These selections are evaluated as seed and pollen parents and progeny are re-evaluated as described above. Several of the turfgrasses tested in the original experiments have now been adapted in continuous liquid suspension cultures. Salinity is gradually introduced into the liquid cultures and the cells are uniformly exposed to assure that those eventually regenerated are not "escapes" from cell culture. According to current literature reports, interaction between salt adaptation mechanisms and true salt tolerance in regenerated plants has impeded salt tolerant species selection. Loss of regenerative competence in selected cell lines (during suspension) has also been experienced in our work.

The WPMC system developed for the ST screening trials has now been adapted to screen for the morphological responses of turfgrasses cultivars to dinitroaniline herbicides in the root zone. Very little conclusive evidence has been accumulated on the morphological response of turfgrass root systems under pressure from herbicides in the root zone, or the losses on non-target turfs due to herbicide. Bluegrass cultivars are currently examined in tissue culture medium supplemented with the preemergent dinitroaniline herbicides. Investigations are split between examination of effects on germination, and investigation of effects on established plants. We are designing a microculture apparatus which will allow us to rigorously evaluate the response of the root system as it encounters varying concentrations of the herbicides. In vitro culture should be an ideal system for these investigations, since it has the unique advantage of allowing the root system to be viewed non-intrusively. The morphology and growth rate of the root systems are both characteristics which can be used to determine the relative tolerance of the line to the herbicide challenge in the field.