

Long-Term Preservation of Clonally Propagated Turfgrass Species

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Objectives:

1. *Develop suitable micropropagation procedures for selected genotypes of bermudagrass, zoysiagrass, saltgrass and buffalograss.*
2. *Develop suitable shoot tip culture media (STCM) for the four species.*
3. *Examine cryopreservation of the four species using vitrification methodologies.*

Clones of all 4 species (bermudagrass, buffalograss, saltgrass, and zoysiagrass) have been established *in vitro* using a ½ strength MS medium with Nitsch & Nitsch vitamins, 5 mg/l of thiamine, 2 mg/l of glycine, 30 g of sucrose with the pH adjusted to 5.7 and 7 g of agar and varying amounts of growth regulators. We now can initiate cultures from the greenhouse with reduced contamination following a disinfestation procedure that includes a prewash in the greenhouse followed by a 15-30 minute wash in soapy water in the laboratory. After 30-60 minutes in a running diionized water rinse, the nodal sections are surface disinfested in 10% bleach with Tween 20 for 15 minutes followed by 3 sterile water rinses. This procedure, sometimes with PPM (a proprietary antimicrobial compound), results in 50% or greater clean cultures. Rapidly growing nodal sections work best and preferably those not established in soil.

We have succeeded in proliferating both buffalograss and saltgrass using the aforementioned media with high levels of BA, 10 mg/l. However, proliferation remains relatively slow requiring 8-12 weeks to develop sufficiently for subculture. We continue to evaluate various levels of BA for subcultured shoots in combination with various other growth substances, primarily low levels of 2,4-D and NAA. We are proliferating these two species in sufficient quantities to provide for cryopreservation studies in the coming year.

Although we have approximately 50 cultures of both bermudagrass and zoysiagrass *in vitro*, proliferation is minimal and requires further research. BA levels of 10 mg/l often cause yellowing and eventual death of cultures of these two species, especially bermudagrass.

Due to the slow progress in developing rapid proliferation of tissue cultures of the species, we started evaluating procedures other than regrowth for determination of viability of frozen buds. We initially looked at browning as an indication of cell/tissue death. Buds subjected to immersion into liquid nitrogen (-196C) without protective treatments and thawed remained green for at 48 hours after freezing. Tissues normally brown, an indication of cell death, after freezing to these temperatures and subsequent thawing. We also looked at the vital stain tetrazolium, TDZ, as an indication of live/dead cells. Again the success was somewhat limited.

We now believe that with increased numbers of cultures of both buffalograss as well as saltgrass that regrowth of frozen buds is once again the preferred procedure. We therefore will begin more detailed freezing studies using regrowth *in vitro* to evaluate survival.

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Clones of bermudagrass, buffalograss, saltgrass, and zoysiagrass are being maintained in a greenhouse as a source for *in vitro* and cryopreservation studies. We have increased these materials vegetatively so that we currently maintain approximately 15 market packs of each species.

We have continued to evaluate disinfestation strategies to optimize procedures. We now can initiate cultures from the greenhouse with reduced contamination following a disinfestation procedure that includes a prewash in the greenhouse followed by a 15-30 minute wash in soapy water in the laboratory. After 30-60 minutes in a running deionized water rinse, the nodal sections are surface disinfested in 10% bleach with Tween 20 for 15 minutes followed by 3 sterile water rinses. The use of PPM, a patented antimicrobial solution, is of some benefit in obtaining clean cultures. However, we have noted that apparently clean culture may become contaminated when cultures are transferred to media without PPM. This would indicate that the PPM is not eliminating the contamination entirely, in some cases at least, but just preventing growth. The above procedure, however, has resulted in 50% or greater clean cultures. Rapidly growing nodal sections work best and preferably those not rooted into the growing media.

Clones of all four species have been established *in vitro* using a ½ strength MS medium with Nitsch and Nitsch vitamins, 5 mg/l of thiamine, 2 mg/l of glycine, 30 g of sucrose with the pH adjusted to 5.7 and solidified with 7 g of agar and varying levels of growth regulators. We have succeeded in proliferating both buffalograss and saltgrass using the above media with high levels of BA 10 mg/l. However, proliferation is relatively slow requiring approximately 8-12 weeks to develop. We currently have over 80 cultures of each of these species and are subculturing the proliferating cultures onto media with the same or reduced levels of BA and with or without NAA/2,4-D at varying levels as well. We are proliferating the cultures in sufficient quantities to provide for cryopreservation studies in the coming year.

Although we have approximately 50 cultures of both bermudagrass and zoysiagrass *in vitro*, proliferation is minimal and requires further research. The nodal sections of bermudagrass does proliferate but only results in 2-3 growing shoots. This is insufficient for use in the cryopreservation studies. The zoysiagrass shows little if any proliferation. At high levels of BA 5/10 mg/l the bermudagrass yellow and eventually die. Zoysiagrass shows yellowing as well but tends to persist much longer in culture.

Since the proliferation of the turfgrass species were slower than we anticipated in tissue culture we began to look at methods other than regrowth in the test tube for determination of viability of frozen buds. Since small pieces of tissue will be used in the cryopreservation process and these pieces cannot regrow by simply planting in soil, we must find some other way of

determining viability after freezing at liquid nitrogen temperatures. If we can effectively determine viability via another procedure we may proceed with various cryopreservation treatments while continuing to develop protocol for proliferation and growth of buds of the 4 turfgrass species in tissue culture.

We initially looked at browning as an indication of cell/tissue death. Buds and associated tissues of all four species were subjected to direct immersion into liquid nitrogen (-196C) without protective treatments. Tissues were allowed to thaw at room temperatures and examined at 15 min, 30 min, 60 min, 2 hrs, 4 hrs, 24hrs and 48 hrs. Browning commonly occurs in plant tissues that have been damaged or killed. Plant tissues remained green during this time with minimal differences between control, no freezing, and treated, frozen at -196 C. Based on these results we decided to look at vital stains as a means of determining viability.

We looked at tetrazolium staining as a means of determining viability next. When the cells stain red after soaking in the stain we can say the tissues are viable. We therefore subjected plant nodal sections to freezing and compared staining results with a nonfrozen control. The results were mixed and we therefore abandoned this procedure as well. Since we are now getting sufficient cultures of buffalograss to start cryopreservation tests on we will use regrowth of tissue cultured nodal sections for our test to determine viability.

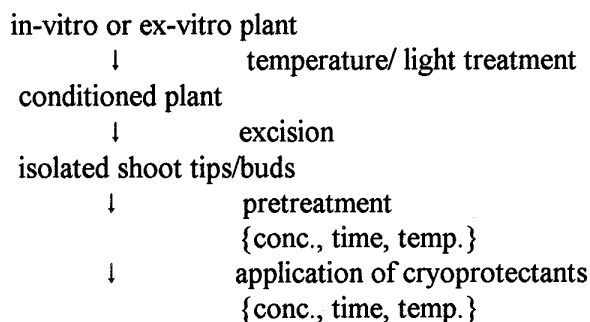
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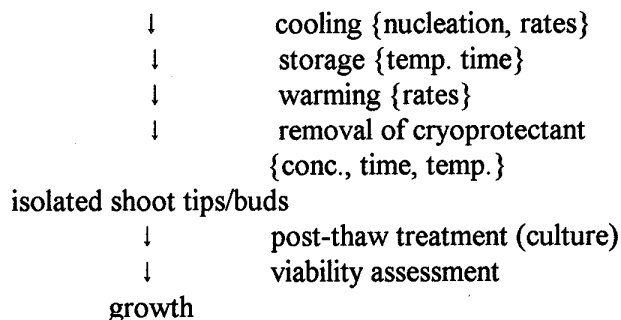
Cryopreservation studies.

Useful micropropagation procedures have been developed for buffalograss and saltgrass such that cryopreservation studies can be initiated. Further studies to develop a more proliferative system in zoysiagrass and bermudagrass are needed before cryopreservation tests can be performed.

The general strategy is to perform cryopreservation tests on the grass which proliferates best in culture and can be recovered from isolated shoot tips. We anticipate that cryopreservation methods that work well for one grass can be used as a general method for application to the other grasses as proliferation and recovery systems are improved and refined. This method may need to be altered in specifics (duration of exposure, for example), but should provide a logical starting point for the other species.

The general flow diagram for cryopreservation can be examined where suitable material is available:





The sequence of studies we intend to pursue involve the following:

1. Develop a shoot tip recovery medium for buffalograss and saltgrass. Recovery media are often different from the micropropagation medium because of the small size of the explant (shoot tip). Growth regulator type and concentration are the major variables. We also intend to pursue using either a tetrazolium or fluorescein test as a 'quick' indicator of viability after cryotreatments for shoot tips derived from in-vitro or pot plants.

2. Examine cryopreservation of isolated shoot tips using vitrification methodologies:

Solution-based vitrification:

Since there are a number of variables (see above), we will first examine pretreatment and cryoprotectant applications using a standard cooling and warming protocol as developed for other non-grass species. The major pretreatments we will examine are kinetic and concentration exposures to selected organics (sugars, proline, sugar-alcohols) and cryoprotectant treatments of PVS2, a mixture of high osmotic components known to be useful for a range of cell types. The major variables are time of exposure and concentration gradients.

Encapsulation-dehydration vitrification:

Again, based upon utility in other systems, this procedure warrants examination. Pretreatment tests would be similar to the above. Extent of desiccation is the major variable.

3. Other studies:

a. Cooling/warming studies:

After levels of viability have been defined in the above tests, certain conditions will be examined in an attempt to optimize recovery. Cooling rate has influenced survival in several systems and will be varied to determine influence on survival and growth.

b. Stock plant growth regime:

Initial studies will be with actively growing cultures, but some plant treatments may improve survival. These would include temperature and photoperiod but may also involve spray applications of growth inhibitors or amino acids.

Tissue culture studies.

Tissue culture studies will focus on continued development of proliferation systems for bermudagrass and zoysiagrass. Growth regulators will be evaluated with a focus on NAA, 2,4-D and thiodiazuron.