To the Graduate Council:

I am submitting herewith a thesis written by Renée Sarah Albertine Fribourg entitled "Improvements of Sporophyte Culture and Comparison of Salinity Tolerance in Three Strains of *Ceratopteris richardii*." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Dr. Leslie Hickok, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

IMPROVEMENTS OF SPOROPHYTE CULTURE AND COMPARISON OF SALINITY TOLERANCE IN THREE STRAINS OF *CERATOPTERIS RICHARDII*

A Thesis

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Degree

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ABSTRACT

Increasing soil salinity on cultivated lands under irrigation is a significant problem confronting world crop production. Due to the multigenic basis of the trait, and problems associated with the regeneration of tolerant plants from tolerant cell cultures, there has been limited progress in the development of salt tolerant crop plants. The use of whole plant mutagenesis and selection for single gene mutants conferring salt tolerance would be useful in studying the genetics associated with the trait. This study documents the selection of salt tolerant mutants in sporophytes of the fern *Ceratopteris richardii*. In an effort to improve general sporophyte culture, several aspects of *in vitro* culture were investigated and a comparative study of 'optimized' culture conditions and NaCl tolerance was also performed. In addition, the feasibility of completing the life cycle under *in vitro* conditions was investigated.

The selection of a mutant (β2-1), conferring tolerance to NaCl was successful. The mutant strain clearly exhibited a higher level of tolerance relative to the parental strain (Nα23-14) and the wild type. Optimized culture conditions significantly enhanced quantitative tolerance on 65 and 80 mM NaCl for each strain tested. This tolerance was transmitted from the M1 to the M2, and from the M2 to the M3 generations, which is strong evidence of a genetic basis. However, because of qualitative variability in

tolerance, genetic characterization of the mutant was not possible.

The improvement of general sporophyte culture included investigations of solidifying agents, light intensity, temperature and nutrient media. Both root and shoot growth were significantly enhanced on washed Difco Bacto™ agar. High light intensities ($>100\ \mu mol\ m^{-2}\ s^{-1}$) were inhibitory to sporophyte growth. Maximum growth appeared to occur at a temperature of 27.6°C. However, because this study was not replicated, the significant difference seen cannot be unequivocally associated with the variable of temperature. The use of 1/2X DeGreef and Jacobs™ nutrient media supported healthy sporophyte growth in both short (18 days) and long term (3 months) cultures when compared to the other medium tested. A comparative study of improved vs traditional culture conditions indicated the improved conditions significantly enhanced sporophyte growth. Treatment with 5.0×10^{-3} and 10^{-2}M filter sterilized GA_3 accelerated sporangial development. However, efforts to complete the life cycle under *in vitro* conditions were unsuccessful.

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LIST OF ABBREVIATIONS

DFS	Days From Sowing
DFT	Days From Transfer
DJ 1X	De Greef and Jacobs basal salt mixture (Full strength)
DJ 1/2X	De Greef and Jacobs basal salt mixture (Half strength)
DKW 1X	DKW/Juglans plant tissue culture medium (Full strength)
DKW 1/2X	DKW/Juglans plant tissue culture medium (Half strength)
EMS	Ethyl Methane Sulfonate
HFS	Hours From Soaking
KNUD	Knudson's basal salt mixture
MS 1X	Murashige and Skoog modified basal salt mixture (Full strength)
MS 1/2X	Murashige and Skoog modified basal salt mixture (Half strength)
P/TC	Parker/Thompson nutrient media
S & H	Schenk and Hildebrandt basal salt mixture
U-BAC	Unwashed Difco Bacto™ agar
U-H	Unwashed Hazelton™ agar
U-PHY	Unwashed Phytagel™
W-BAC	Washed Difco Bacto™ agar
U-H	Unwashed Hazelton™ agar

I. INTRODUCTION

This thesis describes the use of the homosporous fern *Ceratopteris richardii* in a study of the expression of salinity tolerance and improvement of *in vitro* culture of sporophytes. This model system offers an *in vitro* method for studying a variety of physiological and developmental responses (Hickok et al. 1995, Warne et al. 1995). The advantages of the use of *Ceratopteris* are principally associated with various features of the life cycle. Reproduction occurs sexually through meiotic production of haploid spores, which can continue throughout the year under greenhouse conditions. In culture, spores germinate and develop into meristic, bisexual, cordate-shaped gametophytes or ameristic, spatulate male gametophytes. Cordates, which are heart-shaped gametophytes possessing 2-4 archegonia, can be self-fertilized (intragametophytic selfing) to generate homozygous sporophytes, or cross-fertilized (intergametophytic crossing) to generate specific hybrid combinations. Conventional mutagenesis using X-rays or chemical agents can be used to mutagenize large numbers of haploid spores which can be easily screened for desired traits by sowing on agar-solidified media containing a selection agent. Mutant strains are easily comparable to the wild type strain which exhibits sensitivity to a number of agents. Because the gametophytes are haploid, both dominant and recessive mutations can be detected directly (Hickok et al., 1995). To date, most studies have involved the gametophytic phase of the life cycle. The diploid

sporophyte phase has been primarily used for spore production under greenhouse culture.

In contrast to the forementioned emphasis on gametophytes, this project focused primarily on the sporophyte generation. Two main objectives were addressed. First, because of the complexities and limited success associated with the generation of salt tolerant plants in other genera of vascular plants, this system was used to specifically select for salt tolerance in sporophytes. Second, because there are few studies concerning optimization of culture conditions for *Ceratopteris* sporophytes, several aspects of *in vitro* culture were investigated in an effort to improve general sporophyte culture. Also, a comparative study of 'optimized' culture conditions and NaCl tolerance was performed.

Salinity Tolerance

Increasing soil salinity on cultivated lands under irrigation is a significant problem confronting world crop production. Inadequate rainfall in these areas prevents leaching of soluble salts, which at low concentrations suppress plant growth, reducing yields, and at high concentrations are inhibitory to growth (Shannon, 1984). Approaches to the problem have involved efforts to reduce soil salinity by physical means as well as traditional breeding approaches to enhance the salinity tolerance of crops.

The use of conventional breeding methods for increasing salt tolerance in

crop plants has had limited success. Several studies have shown continuous variation of the character and hence a multigenic basis for the trait (e.g., Furr and Ream, 1969; Norlyn, 1980). Dvorák and Ross (unpublished data referenced in Dvorák, 1985) showed the expression of salt tolerance of *Elytrigia elongata* (Host) in the background of the salt susceptible wheat cultivar 'Chinese Spring' to be dependent on complementary interactions among genes on different chromosomes. Gene interaction and qualitative variability associated with environmental effects as well as time, have been obstacles in the development of distinctive phenotypic markers for salinity tolerance which could be used in a breeding program (Epstein and Rains, 1987). Consequently, limited progress has been made in identifying specific genes associated with the trait (Epstein et al., 1980; Cushman et al., 1990). In addition to these complications, the ability to screen large numbers of progeny for the desired genotype is both costly and time consuming. Together, these factors have contributed to the limited advancement in our understanding of salinity tolerance.

Cell culture techniques have been successful in the selection of several NaCl tolerant cell lines (Dix and Street, 1975; Croughan et al., 1978; Hasegawa et al., 1980; Smith and McComb, 1983; Binzel et al., 1985; Bressan et al., 1987; Winicov et al., 1989) and are now widely used in salinity studies. It is argued that cell culture offers several potential advantages over conventional selection and breeding methods; these

include: (a) characterization at the cellular level of physiological and genetic markers associated with salt tolerance; (b) reduction of complications associated with morphological variability found in differentiated plant tissues; (c) the control of environmental and nutrient conditions for manipulation of plant cell populations; (d) the ability to manipulate large numbers of cells; and (e) utilization of procedures such as somatic hybridization, gene transfer, and mutant induction and selection (Epstein and Rains, 1987). However, to be an effective tool in elucidating the genetics of salt tolerance, the character selected for at the cellular level must be expressed at the whole plant level and transmitted to the next generation through meiosis (Dracup, 1991; Sumaryati et al., 1992).

In spite of their promise, cell culture approaches have had limited success in regenerating and maintaining salt tolerance at the whole plant level. For instance, plants regenerated from tolerant alfalfa cell lines were characterized by having extensive somaclonal variation and exhibited aberrant morphologies (Bingham and McCoy, 1986; McCoy, 1987a). In addition, other regenerates were not significantly more tolerant than the original population (Smith and McComb, 1983) or showed an almost inverse relationship between whole plant and cellular tolerance (McCoy, 1987b). Other characteristics observed in plants regenerated from long term callus cultures of alfalfa include slow and stunted growth, and susceptibility to disease (Stavarek et al., 1980). Shoot cultures regenerated from salt-

tolerant cell lines of sugarbeet, canola, Chinese cabbage and tobacco failed to show evidence of any salt tolerance or gradual adaptation to salt (Chandler et al., 1988). Possible reasons for the discrepancy between tolerance in cell lines and altered expression in regenerated plants are, (a) cellular tolerance may not be adequate to affect the integrated functioning of whole plant responses to high NaCl, particularly solute transport from root to shoot; and (b) the expression of traits selected in cell culture may not be uniformly expressed in whole plants since this may depend on the hormonal and nutritional environment of individual cells, which differs between cells in culture and in whole plants (Dracup, 1991).

In several studies, using tobacco, salt tolerant plants have been successfully regenerated from cultured cell lines. However, in one case, the use of a stepwise selection process may have resulted in multigenic mutants which were not suitable for Mendelian-based analysis of inheritance (Nabors et al., 1980). In another attempt, segregation ratios of seeds obtained from regenerated plants of *Nicotiana plumbaginifolia* did demonstrate a genetic basis for salt tolerance through a single dominant Mendelian gene. However, the salt resistant plants were male-sterile, perhaps an effect of the high salt concentrations in the media (Sumaryati et al., 1992). In other studies, plant regenerates from salt tolerant cell lines in tobacco exhibited a reduced growth rate and chromosomal aberrations such as hexaploidy (Bressan et al., 1985).

In contrast to the numerous examples of limited success in plant regeneration from culture, a recent study by Winicov (1991) reported successful regeneration of tolerant alfalfa plants from salt tolerant cell lines. In this report, the regenerated plants exhibited a stable salt tolerant phenotype, showed vigorous growth and were self fertile. F2 progeny obtained by selfing the tolerant plants showed that overall cellular tolerance on 1% NaCl was maintained in 60% of the progeny, suggesting the presence of a dominant mutation in the regenerated plants. F2 seedlings also exhibited >50% tolerance. However, cellular and whole plant tolerance was less than the expected 75% for a single dominant trait. Reasons for the discrepancy were postulated as being due to: a) a necessary interaction of the segregating locus with other independently segregating loci, or B) the natural distribution of individual plant growth responses influences the growth measurements ± NaCl.

In an attempt to identify specific genes associated with salinity tolerance in whole plants, Saleki et al. (1993) used the model plant *Arabidopsis* to select salt tolerant individuals generated from mutagenized seeds. Three mutant strains (RS17, RS19 and RS20) were selected for their capability to germinate under high saline conditions. Two of these, RS17 and RS20, could germinate on media containing up to and including 225 mM NaCl whereas RS19 was resistant to 175 mM. The wild type could not germinate at concentrations >125 mM NaCl. F1 progenies of crosses between wild

type and mutants indicated all mutations were recessive to wild type alleles. Complementation analysis indicated the traits as being distinct genes in each line. F2 segregation data for RS17 and RS20 fit the model of a single gene pattern of inheritance, whereas for RS19 data was problematic. Although these mutants exhibited increased tolerance to NaCl, they exhibited even greater resistance to osmotic stress imposed by mannitol, suggesting they were primarily osmotolerant. In addition, when seedlings were exposed to salinity stress there was no difference in tolerance between mutant and wild type strains, indicating restriction of the tolerant phenotype to the germination stage.

In another approach, Hickok et al., (1987) described the use of the homosporous fern *Ceratopteris richardii* for studying the physiological and genetic aspects of salt tolerance. This system has successfully been used to select three unlinked single gene mutations designated *stl1*, *stl2* and *stl3* (Warne and Hickok, 1987a; Hickok et al., 1991), each of which confers tolerance to NaCl in the gametophyte stage. Of these, the *stl2* mutation confers a high level of tolerance, up to 275 mM NaCl. In contrast to gametophytes, studies of sporophytes carrying the *stl2* mutation showed NaCl concentrations above 60 mM were restrictive to growth. Because of the low level of sporophytic tolerance associated with the *stl2* mutation, a separate study to specifically select for enhanced sporophytic tolerance could be of significant value to better understand the physiological and

genetic aspects of whole plant tolerance. Such a study comprises the first aspect of this research project.

Culture Improvements

During the course of pursuing the salinity studies mentioned above, unexpected variability in sporophyte responses was noticed. Such variability hampered accurate interpretation of the data obtained. Therefore, it was considered essential to improve and standardize experimental conditions for *in vitro* sporophyte culture in order to identify and control possible sources of variation. Hence, the second part of this research project involves an investigation into the improvement of general sporophyte culture under *in vitro* conditions. The following variables were considered: solidifying agents, light intensity, temperature and nutrient media. Although a number of studies have documented the effects of many of these variables on fern gametophyte culture (Mohr 1956; Miller and Miller, 1961; Sussman 1965; Mahlberg and Yarus, 1977; Dyer 1979; Warne and Lloyd, 1980), few studies have been reported on their effects on sporophytes (Mahlberg and Yarus, 1977. After optimization of the above variables, a comparative study of three strains of sporophytes differing in their level of salt tolerance was made.

As a final component of this research project, the feasibility of completing the entire life cycle under *in vitro* conditions was investigated.

The life cycle (from spore to spore) of *Ceratopteris richardii* is approximately 120 days. Spores germinate in 2-3 days from sowing (DFS), and develop into sexually mature gametophytes in 6-9 DFS. Embroys develop into mature sporophytes ready for transfer to the greenhouse in about 25-30 DFS. The remainder of the life-cycle is spent in the greenhouse where the sporophyte initially produces vegetative fronds with subsequent fronds becoming increasingly fertile over time. Because the ability to complete the life cycle under *in vitro* conditions would be advantageous to the use of *Ceratopteris* as a model system, experiments were carried out to determine if this developmental progression from vegetative to fertile fronds (i.e. the heteroblastic leaf sequence) could be accelerated.

Past studies have suggested that gibberellic acid could be useful in reducing the time necessary for fertile frond production. For instance, Scurfield and Moore (1958) found that when seedlings of the *Eucalyptus* species were treated with gibberellic acid the adult type of leaf developed earlier. In another report, Allsop (1962) studied the effects of gibberellic acid on morphogenesis in the heterosporous fern, *Marsilea*, by measuring the degree of leaf segmentation. Here the application of gibberellic acid was shown to decrease the rate of leaf formation (number of leaves per plant) but increase the rate of heteroblastic development. However, in this study the media was supplemented with glucose and the effects of gibberellic acid depended on the glucose concentration of the medium. For example, 4%

glucose increased the rate of heteroblastic leaf formation when compared to 2% glucose. Also, the observed increased rate of development in media supplemented with 4% glucose was not raised further by the addition of gibberellic acid whereas in the 2% glucose there was a significant increase. More recently, Stein (1971) reported that gibberellic acid could on a precise and predictable time table shorten the life cycle of *Ceratopteris thalictrodes* by inducing the transition from vegetative to fertile fronds. However, in this report spore viability was $\leq 50\%$. These findings promoted an investigation into the effect of gibberellic acid on the heteroblastic leaf sequence in *Ceratopteris richardii* cultured under *in vitro* conditions.

II. MATERIALS AND METHODS

Spore Sources

Spores from two different strains of *Ceratopteris richardii* were used; the wild-type, Hn, collected on 11/85 and Nα23-14 collected on 10/85. Strain Nα23-14, contains the nuclear gene mutation designated *stl2* (Hickok et al., 1991) which confers a high level of NaCl tolerance to gametophytes and a moderate level of NaCl tolerance to sporophytes.

General Culture Conditions

To establish cultures, spores were weighed and then soaked in distilled water for 15 minutes in a 15 ml centrifuge tube. After 15 minutes the water was removed using a pasteur pipet and spores were sterilized using a 0.875% sodium hypochlorite solution (1:5 dilution of commercial bleach) for 3 minutes. The sodium hypochlorite was removed and spores were rinsed twice with sterile distilled water. Sterilized spores were sown axenically onto 100 x 15 mm petri dishes containing agar-solidified (1% w/v) nutrient media (pH 6.0). Spores were spread using a wire loop. In multispore cultures, general spore sowing densities were 1.0 mg per 100 x 15 mm plate. Petri plates were placed in 24" x 48" opaque plastic trays covered with a clear plastic dome. Standard cultures were maintained under constant culture room conditions of 28° ± 1°C and 24 h. of 80 μmol $m^{-2}s^{-1}$

photosynthetic active radiation (PAR) from 40W cool white fluorescent tubes. Typically, spores germinate in 2-3 DFS and sexually mature gametophytes develop in 6-9 DFS. Sporophytes were generated at 6-9 DFS by flooding plates with sterile distilled water to stimulate the release of spermatozoids for fertilization, a technique generally referred to as 'intergametophytic selfing'. Mature sporophytes, having well developed roots and ca. 10 leaves, were transferred to a greenhouse for production of spores. The potting media was a 2:1:1 mixture of peat, vermiculite and perlite with pH adjusted to 6.5 by adding agricultural lime at a rate of 100 g/ 10 gal. potting mix (Hickok et al., 1995).

Sporophyte Growth Measurements

Sporophytic responses to various treatments were determined qualitatively by visual inspection and quantitatively by fresh and dry weight measurements. Sporophytes at the one-leaf stage were transferred to treatment medium and at 18 days from transfer (DFT) were removed from treatment plates, blotted with Bibulos™ paper and weighed to the nearest milligram for fresh weight determination. Sporophytes were then dried for 24 hr. at 80°C, cooled in a desiccator for 15-30 minutes, and dry weight taken. In most cases root and shoot fresh and dry weights were taken separately.

Sporophyte Tolerance To NaCl

Selection

For a flow chart of the following selection scheme please see Figure 1. Chemical mutagenesis was accomplished by soaking 160 mg of Nα23-14 spores (about 2.0 x 10^5 spores) in a 0.2 M solution of ethyl methane sulfonate (EMS) made in 0.1 M phosphate buffer, pH 7.0 for 36 hr. Mutagenized spores were washed three times and sown axenically on 100 x 15 mm plates (density 2 mg spores/plate) containing mineral nutrient medium supplemented with 80 mM NaCl. Sporophytes were generated by flooding the plates with sterile distilled water (intergametophytic selfing) at 10-14 days from sowing (DFS). After several weeks of culture, putative tolerant sporophytes were visually selected and transferred to fresh 80 mM NaCl supplemented media for further observation. Three replicates of this screening procedure were performed. Sporophytes that maintained tolerance on 80 mM NaCl were designated as M1 generation putative mutants. These were transferred to unsupplemented media for pre-greenhouse culture, then potted in a standard soil mix and moved to a greenhouse for production of M2 generation spores.

M2 spores were collected and ripened with ethrel™ (Amchem products, Inc), for 6 days at 37°C to increase germination rate (Warne and Hickok, 1987b). Spores were then axenically sown on unsupplemented media (~2 mg per 100 x 15 mm plate) and at 10 DFS gametophytes were transferred

Figure 1. Flow chart for the mutagenesis and selection of enhanced tolerance to NaCl in sporophytes of *Ceratopteris richardii* using the genetic background of the N*α*23-14 strain.

Nα23-14 ← EMS mutagenized

↓

Mutagenized spores were sown on 80 mM Nacl, interselfed and resulting M1 sporophytes qualitatively screened for tolerance.

↓

M1 generation sporophytes which exhibited tolerance in multispore cultures were transferred to fresh 80 mM NaCl plates and further observed for tolerance.

↓

M1 sporophytes that maintained tolerance were coded as β1 through β14 and transferred to the greenhouse for production of M2 generation spores.

↓

M2 spores were sown on unsupplemented media and randomly selected M2 gametophytes were transferred to 80 mM NaCl and intraselfed, resulting in putative homozygous M2 sporophytes.

↓

Tolerant:Sensitive ratios of M2 putative homozygous sporophytes were assessed. Those conferring tolerance were coded as β1-1, etc., and transferred to the greenhouse for production of M3 generation spores.

↓

M3 spores were sown on unsupplemented media, interselfed and one-leaf stage sporophytes were transferred to 80 mM NaCl and assessed for tolerance.

Figure 1

to 100 x 15 mm plates (~ 5 per plate) containing 80 mM NaCl supplemented media and individually watered for the production of M2 generation homozygous sporophytes (intragametophytic selfing). Because there were several gametophytes per plate, there was a slim possibility that cross fertilization between gametophytes might have occurred. Therefore, resulting sporophytes were considered putative homozygotes. Sporophytes that appeared tolerant were designated as M2 homozygotes and were transferred to the greenhouse for production of M3 generation spores.

M3 spores were collected and ripened as previously described and were axenically sown on unsupplemented media (~2 mg per plate). At 10-14 DFS plates were flooded for intergametophytic selfing. One-leaf stage M3 sporophytes were transferred to 80 mM NaCl supplemented media and screened to determine the percentage of tolerance exhibited within each strain. M2 parental strains exhibiting >50% tolerance in M3 generation sporophytes were maintained under greenhouse conditions for further production of M3 spores.

NaCl Dose Response

Multispore cultures were established by sowing M3 spores of the selected M2 parental strains according to standard procedure. Sporophytes were generated by flooding the plates with sterile distilled water (intergametophytic selfing) at 10-14 DFS. One-leaf stage sporophytes were

transferred to treatment media plates containing various NaCl concentrations (10 sporophytes per 100 x 15 mm petri plate). In the first dose response concentrations of 40, 50, 55, 65, 75 and 80 mM NaCl were used. Sporophytes remained on treatments for a period of 18 to 21 days. In the second dose response 0, 35, 55, 75, 95 and 115 mM NaCl were used. Treatment time was 18 days. The shorter time was decided upon in order to avoid possible enhancement of necrosis from nutrient deficiencies. Determination of 'tolerant' sporophytes was done by visually screening for the appearance of healthy shoot and root growth and lack of excessive necrosis. Those sporophytes exhibiting little or no growth were considered 'sensitive'. Prior to removal from treatments for fresh and dry weight measurements, photographs of sporophytes in treatment dishes were taken.

Sporophyte Culture Improvements

Solidifying agents

The following study was designed to assess the effect of solidifying agents on sporophyte growth as well as NaCl tolerance. Treatments were a factorial arrangement of five solidifying agents [1% w/v of washed and unwashed Hazelton™ agar, washed and unwashed Difco Bacto™ agar, and Phytagel™ (Sigma)], three salinity levels (0, 65, and 80 mM NaCl) and four strains (Hn, Nα23-14, β2-1, and β3-10). To wash the agar, 10 grams of agar was added to a Buchner funnel lined with Miracloth™ and flushed ca.

10 times with 100 ml aliquots of double-deionized distilled water. To prevent clogging of the funnel it was necessary to change the Miracloth™ half way through washing. Multispore cultures were established axenically on Parker/Thompson nutrient media as previously described. To help synchronize germination rates among strains, sown plates were wraped in aluminum foil and placed in the dark for three days. Sporophytes were generated by flooding the plates with sterile distilled water (intergametophytic selfing). Twenty one-leaf stage sporophytes were transferred to each treatment (10 sporophytes per 100 x 15 mm petri plate). At 18 DFT sporophytes were removed from treatment and fresh and dry weights were taken as previously described. Two replicate plates of each treatment were run simultaneously and data combined for analysis.

Light Intensity

A block design with sampling and fixed treatments within blocks was used to determine the effects of various light intensities and background (tray) color on sporophyte growth. Variations in light intensity were accomplished in two ways. First, the bottoms of each of four culture trays (blocks) were divided into four sections. One covered with white paper, one with aluminum foil, and the remaining two were sections of gray and black trays. Second, two of the blocks were elevated in order to receive higher light intensities. Both direct and reflected light were measured using a LI-

COR Radiation Sensor from Li-COR Inc. Multispore cultures of the wild type (Hn) were established on agar-solidified (washed Difco Bacto™) Parker/Thompson nutrient media according to standard procedure. Sporophyte generation and transfers were performed as previously described (10 transfers per 100 x 15 mm petri dish). At 18 DFT fresh and dry weights were taken as previously described. Two replicate plates of each treatment were performed and the data combined for analysis.

Temperature

A completely randomized design was used to test the effects of various temperatures on sporophyte growth. Temperatures within culture trays were monitored using temperature probes coupled to a LI-1000 Datalogger from Li-Cor Inc. Treatment temperatures included 26.5 ± 0.09°C, 27.6 ± 0.13°C, 28.5 ± 0.21°C, 29.6 ± 0.04°C, 30.3 ± 0.32°C. Multispore cultures of the wild type, (Hn), were established on agar-solidified (washed Difco Bacto™) Parker/Thompson nutrient media according to standard procedure. Sporophytes were generated by intergametophytic selfing as previously described. One-hundred transfers of one sporophyte per 60 x 15 mm petri dish were made. Dishes were randomly distributed among treatments. At 18 DFT fresh and dry weights were taken as previously described. Twenty replicate plates of each treatment were performed and data combined for analysis.

Nutrient media

A randomized block design was used to test the effects of various media on sporophyte growth. Nutrient compositions of all tested media are shown in the Appendix, Table 10. Multispore cultures of the wild type, (Hn), were established on agar-solidified (washed Difco Bacto™) Parker/Thompson media. Due to the large amount of agar needed for this experiment a different method of agar purification was employed (Binns and Meins, 1979). This method used successive washings with 100 ml aliquotes of double deionized distilled water until the eluant was clear (ca. 1 L), 95% ethanol (300 ml) and HPLC grade acetone (300 ml), followed by air drying for 24 hrs. This method enabled the use of a standard stock of purified agar in multiple experiments. Sporophytes were generated by intergametophytic selfing as previously described. Two hundred fifty-two transfers of one sporophyte per 60 x 15 mm petri dish were made. Plates were evenly distributed among each of nine blocks. At 18 DFT fresh and dry weights were taken as previously described. Eighteen replicate plates of each treatment (2 replicates per block) were performed and data combined for analysis.

Gibberellic acid

The potassium salt of GA_3 (purity 50%, Sigma), was used in the following study. Treatments were a factorial arrangement of five GA_3

concentrations (10^{-2}, 5.0 x 10^{-3}, 10^{-3}, 5.0 x 10^{-4}, and 10^{-4} M) and three methods of preparation (media incorporated, filter sterilized and autoclaved). For filter sterilized and autoclaved treatments, a 10^{-2} M GA_3 stock solution was prepared using double deionized distilled water as the solvent. The remaining concentrations were prepared by serial dilutions. For the media incorporated treatments, GA_3 powder was directly added to prepared media in order to avoid significant volume displacement. Multispore cultures were established on agar-solidified (washed Difco Bacto™) 1/2X DeGreef and Jacobs nutrient media. Sporophytes were generated by intergametophytic selfing as previously described. Thirty transfers of one sporophyte per Magenta™ jar containing 75 ml agar-solidified (washed Difco Bacto™) DeGreef and Jacobs nutrient media were made. Thirty μl of the filter sterilized and autoclaved solutions were applied once a week to the same leaf (first leaf) for a period of five weeks. At six weeks sporophytes were observed for number of leaves, leaf segmentation and presence of sporangia. Because this experiment was of a preliminary nature there were only two replicates of each treatment.

Comparison of Culture Methods and Salinity Tolerance

A block design with fixed treatments within blocks was used to determine the effect of the previously used culture method, 'old', and the optimized culture method, 'new', on sporophyte growth. In addition, the

effect of both methods on salinity tolerance of three strains was tested. Treatments were a factorial arrangement of two culture methods ('old' and 'new'), three salinity levels (0, 65 and 80 mM NaCl), and three strains (Hn, Nα23-14 and β2-1). The 'old' method consisted of Parker/Thompson nutrient media solidified with unwashed Difco-Bacto™ agar, and a black background. The 'new' method consisted of 1/2X DeGreef and Jacobs nutrient media solidified with washed Difco-Bacto™ agar, and a white background. Blocks were formed by trays with the bottom of each tray divided into two sections (black and white). Multispore cultures of each strain were established on each of the treatment media according to standard procedure. Sporophyte generation was performed as previously described. Sixteen transfers of one sporophyte per 60 x 15 mm petri dish were made for each strain on each treatment. Plates of each strain were divided among four blocks (each block receiving 4 replicate plates of each treatment) and at 18 DFT shoot and root fresh and dry weights were taken as previously described.

Statistical Analysis

Data were analyzed by General Linear Models (GLM) procedures (SAS), and means were separated using Duncan's Multiple Range tests at the 5% level.

III. RESULTS

Selection For Enhanced Sporophytic Tolerance To NaCl

The screening process resulted in the selection of fourteen putative tolerant (as described in Materials and Methods, NaCl Dose Response), M1 sporophytes, identified as Nα23-14-β1 through Nα23-14-β14. Intragametophytic selfing of M2 gametophytes (see Materials and Methods), derived from spores of each of the fourteen M1 sporophytes resulted in fourteen families of putative homozygous sporophytes. Each family was screened for sensitive and tolerant types on 80 mM NaCl. The percent tolerance within these families ranged from 8-50%. A total of eighty-six putative tolerant homozygous M2 sporophytes were recovered (Table 1) and were designated Nα23-14-β*-* (i.e. Nα23-14-β1-1, etc.). The eighty-six M2 sporophytes were placed in the greenhouse for production of M3 generation spores. Over the course of five months, families of M3 generation sporophytes derived from forty-eight of the M2 families were screened for sensitive and tolerant types on 80 mM NaCl and percent tolerance determined. Of these, two strains, Nα23-14-β3-10 and Nα23-14-β2-1, exhibited 43% and 70% tolerance respectively (Table 2). Because of the higher level of tolerance associated with Nα23-14-β2-*1* this strain was chosen as the primary M2 stock for all subsequent tests and will hereafter be referred to as β2-1.

Table 1. Numbers of putative tolerant sporophyte segregates obtained from M1 parental strains.

M1 Parental Strain Nα23-14-β-*	# of Cordates Transferred to 80mM NaCl and Selfed	# Tolerant Sporophytes(%)	M2 Generation Sporophyte Code Name Nα23-14-β*-*
β1	82	12 (15)	β1-1 to β1-12
β2	16	4 (25)	β2-1 to β2-4
β3	25	11 (44)	β3-1 to β3-11
β4	37	3 (8)	β4-1 to β4-3
β5	35	6 (17)	β5-1 to β5-6
β6	40	8 (20)	β6-1 to β6-8
β7	29	4 (14)	β7-1 to β7-4
β8	11	3 (27)	β8-1 to β8-3
β9	19	6 (32)	β9-1 to β9-6
β10	27	9 (33)	β10-1 to β10-9
β11	12	6 (50)	β11-1 to β11-6
β12	16	3 (19)	β12-1 to β12-3
β13	17	4 (24)	β13-1 to β13-4
β14	19	7 (37)	β14-1 to β14-7
23-14	72	4 (5)	N1 to N4

Table 2. Tolerance of families of M3 generation sporophytes to 80 mM NaCl. M3 spores were obtained from the families of tolerant M2 strains listed in Table 1. Sporophytes were transferred to 80 mM NaCl at the one-leaf stage. *These data where obtained from two separate experiments. In the first experiment, which resulted in 43% tolerance, media was solidified with unwashed Hazelton™ agar. In the second experiment, which resulted in 1% tolerance, media was solidified with unwashed Difco Bacto™ agar.

Table 2

M2 Generation Homozygous Sporophytes	# Sporophytes Transferred to 80mM NaCl	# Tolerant Sporophytes(%)	M2 Generation Homozygous Sporophytes	# Sporophytes Transferred to 80mM NaCl	# Tolerant Sporophytes (%)
β1-1	27	1 (4)	β5-6	28	5 (18)
β1-3	26	5 (19)	β6-1	34	0 (0)
β1-6	26	6 (23)	β6-2	35	0 (0)
β1-8	27	1 (4)	β6-3	36	0 (0)
β1-10	28	1 (4)	β6-5	38	0 (0)
β1-12	13	1 (8)	β6-7	37	0 (0)
β2-1	10	7 (70)	β7-1	24	0 (0)
β2-2	10	4 (40)	β7-4	24	0 (0)
β2-4	10	0 (0)	β8-1	17	0 (0)
β3-1	27	3 (11)	β8-2	34	0 (0)
β3-4	26	1 (4)	β8-3	18	0 (0)
β3-6	28	3 (11)	β9-2	24	2 (8)
β3-7	30	5 (17)	β9-3	24	1 (4)
β3-10*	30	13 (43)	β9-6	24	0 (0)
β3-10*	38	1 (3)	β10-1	38	0 (0)
β4-1	29	0 (0)	β10-2	35	0 (0)
β4-3	31	1 (3)	β10-4	37	0 (0)
β4-4	30	3 (10)	β10-6	36	0 (0)
β4-5	30	7 (2)	β10-7	35	0 (0)
β4-6	30	5 (17)	β11-1	35	2 (6)
β5-1	30	3 (10)	β11-2	37	2 (5)
β5-2	29	2 (7)	β11-3	17	0 (0)
β5-3	28	1 (4)	β11-4	33	0 (0)
β5-4	29	1 (3)	β11-6	35	0 (0)
23-14	37	0 (0)	23-14-N2	43	0 (0)
23-14-N1	38	1 (2)	23-14-N4	38	1 (2)

Sporophyte Dose Response

To characterize the level of tolerance exhibited by β2-1, a comparative dose response of Hn, Nα23-14 and β2-1 was performed. Because all these strains were homozygous, tolerance was determined by generating a family of genetically identical homozygous sporophytes from each strain and determining the number of tolerant sporophytes within each family. The three strains exhibited distinctly different responses to NaCl stress (Figure 2). Although Nα23-14 showed increased tolerance relative to the wild type, β2-1 clearly exhibited a higher level of tolerance. On 80 mM NaCl, more than 60% of β2-1 sporophytes were tolerant, whereas tolerance for Hn and Nα23-14 was reduced by 50% on ~35 and 60 mM NaCl, respectively. Qualitative comparisons of the three strains on 0 and 80 mM NaCl are shown in Figure 3.

In order to quantify the NaCl concentration necessary to reduce tolerance of β2-1 by 50%, a second dose response experiment was performed. The range of NaCl concentrations used in this test was selected based on the results of the previous dose response experiment. These consisted of 0, 35, 55, 75, 95, and 115 mM NaCl. The qualitative tolerance previously exhibited by β2-1 was significantly reduced in this second dose response experiment (Figure 4). Although Nα23-14 and β2-1 clearly exhibit a higher level of tolerance than Hn on 55 mM, 50% reduction of tolerance for both Nα23-14 and β2-1 occurred between 65 and 70 mM NaCl. β2-1 was

Figure 2. Percent tolerant Hn, Nα23-14 and β2-1 sporophytes at 18-21 DFT over a range of NaCl concentrations (0, 40, 50, 65, 75 and 80 mM). (Symbols represent percent tolerant out of 20 transfers of each strain).

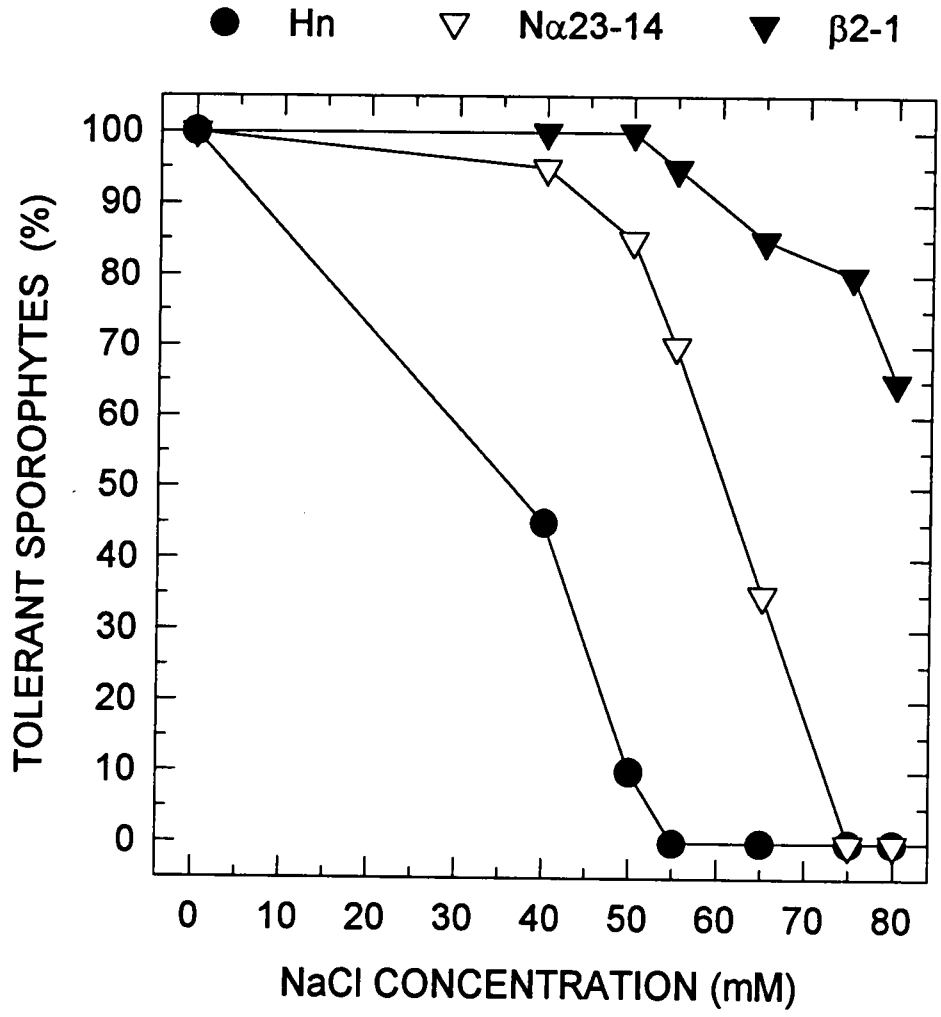

Hn
Nα23-14
β2-1
TOLERANT SPOROPHYTES (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
NaCl CONCENTRATION (mM)

Figure 2

Figure 3. Growth of sporophytes at 18 DFT cultured on 0 and 80 mM NaCl. Hn (A, B), Nα23-14 (C,D) and β2-1 (E,F).

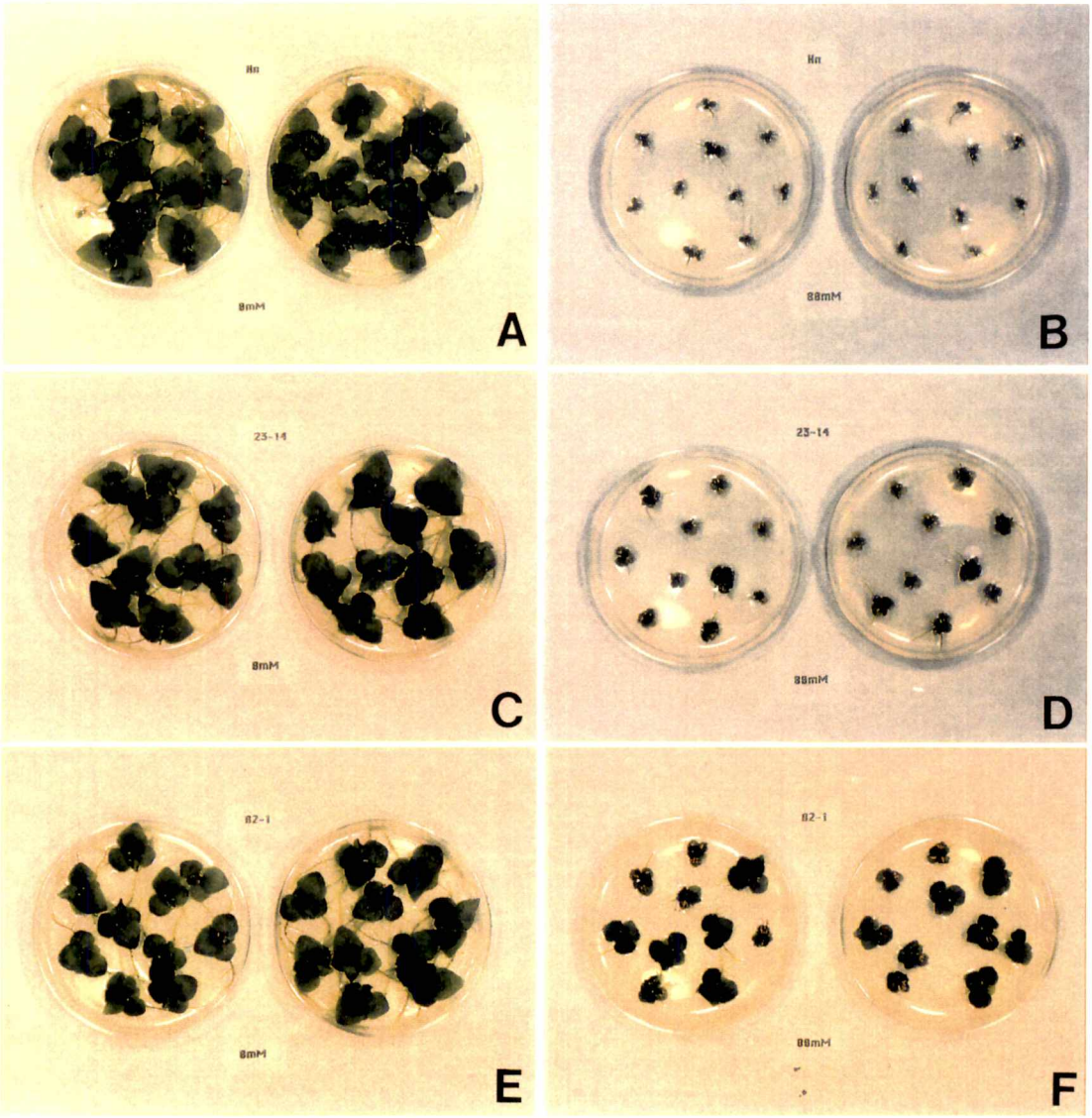


Figure 3

Figure 4. Percent tolerant Hn, Nα23-14 and β2-1 sporophytes at 18 DFT over a range of NaCl concentrations (0, 35, 55, 75, 95 and 115 mM). (Symbols represent percent tolerant out of 20 transfers of each strain).

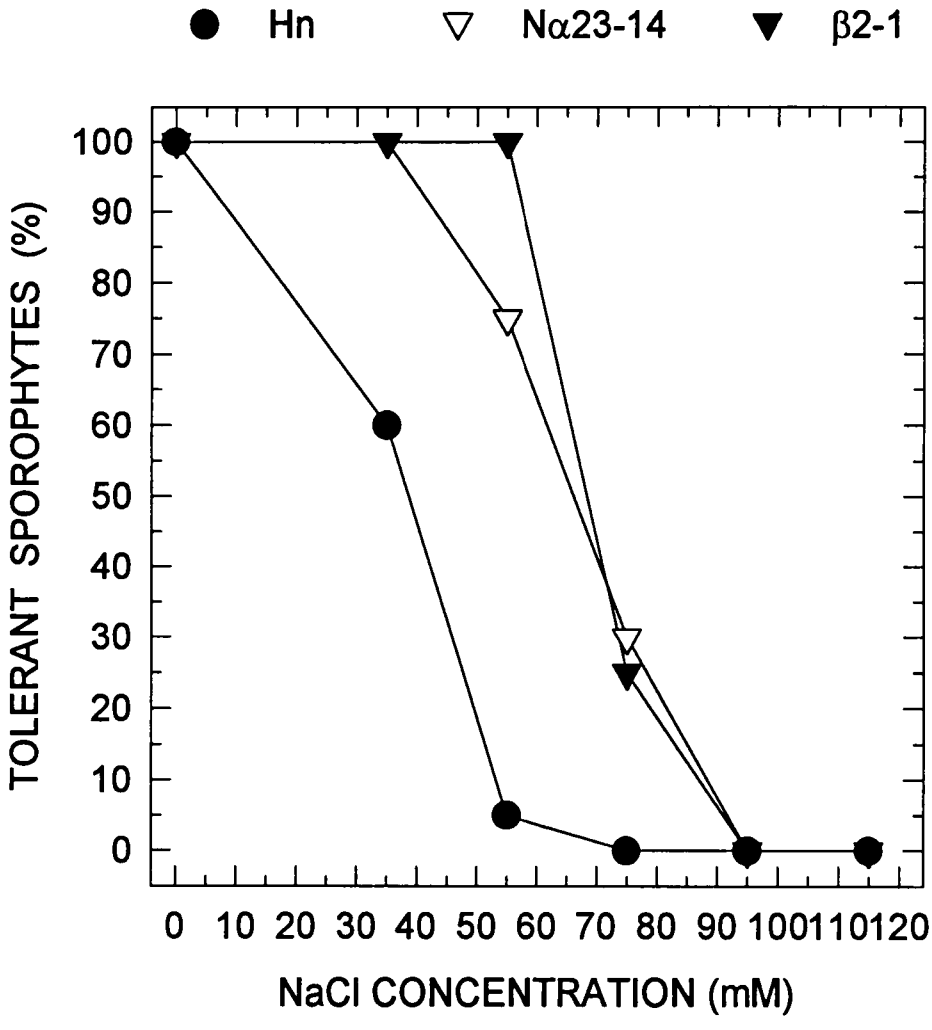

Hn
Nα23-14
β2-1
TOLERANT SPOROPHYTES (%)
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100110120
NaCl CONCENTRATION (mM)

Figure 4

distinct from Nα23-14 only at 55 mM NaCl.

In an attempt to determine the cause of the observed differences between β2-1 responses shown in Figures 2 and 4, five different spore collections of β2-1, collected at different times were tested for percent sporophyte tolerance on 80 mM NaCl. Although there was some variation in the level of tolerance expressed by the different collections, overall percent tolerance of any one collection was not as substantial as previously exhibited (Table 3).

The possibility that the variable results from the β2-1 tests were associated with unexpected temperature fluctuations (i.e. if this mutation was temperature sensitive) was investigated by performing a comparative test of Hn, Nα23-14 and β2-1 cultured under a range of temperatures. Sporophytes were cultured at 24°C, 28°C and 30°C for 18 days after which fresh and dry weights were taken as previously described.

From the data, NaCl tolerance was increased markedly at the lower temperature of 24°C and to a lesser degree at the higher temperature of 30°C (Figure 5). However, this was true not only for Nα23-14 and β2-1 but also the wild type (Hn). In addition, it is important to note that the 0 mM controls of each strain were affected by temperature as well (Figure 6). Growth of Hn was significantly reduced at 24°C and 30°C. Other strains were likewise affected by these temperatures but to a lesser degree.

Examination of quantitative data obtained from the previous three

Table 3. Sporophytic tolerance to 80 mM NaCl at 18 DFT of Hn, Nα23-14 and five separate spore collections of β2-1.

Strain / Collection date	# of One-leaf Stage Sporophytes Transferred to 80mM NaCl	# Tolerant Sporophytes(%)
Hn 11/85	30	0 (0)
Nα23-14 11/89	30	1 (3)
Nα23-14-β2-1 4/92	30	5 (17)
Nα23-14-β2-1 10/92	30	8 (27)
Nα23-14-β2-1 2/93	30	1 (3)
Nα23-14-β2-1 3/93	30	0 (0)
Nα23-14-β2-1 12/93	30	4 (13)

Figure 5. Sporophyte fresh weight at 18 DFT of Hn, Nα23-14 and three β2-1 spore collections cultured on 80mM NaCl under three different temperatures. Data are represented as a percent of the 0mM control at each temperature. (Symbols represent means ± standard error of 20 measurements. Note; in all cases the standard errors are smaller than the symbol size).

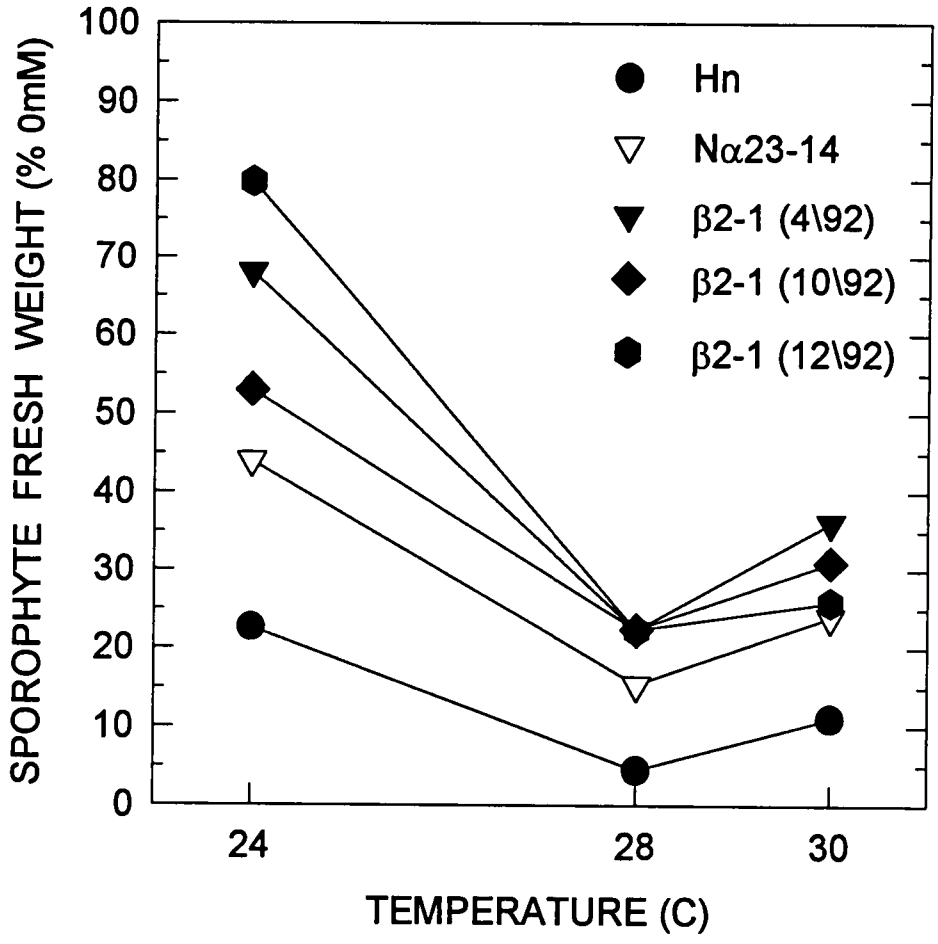

SPOROPHYTE FRESH WEIGHT (% 0mM)
100
90
80
70
60
50
40
30
20
10
0
24
28
30
TEMPERATURE (C)
Hn
Nα23-14
β2-1 (4\92)
β2-1 (10\92)
β2-1 (12\92)

Figure 5

Figure 6. Sporophyte fresh weight at 18 DFT of Hn, Nα23-14 and three β2-1 spore collections cultured on 0mM NaCl under three different temperatures. (Symbols represent means ± standard error of 20 measurements. Note; in all cases the standard errors are smaller than the symbol size).

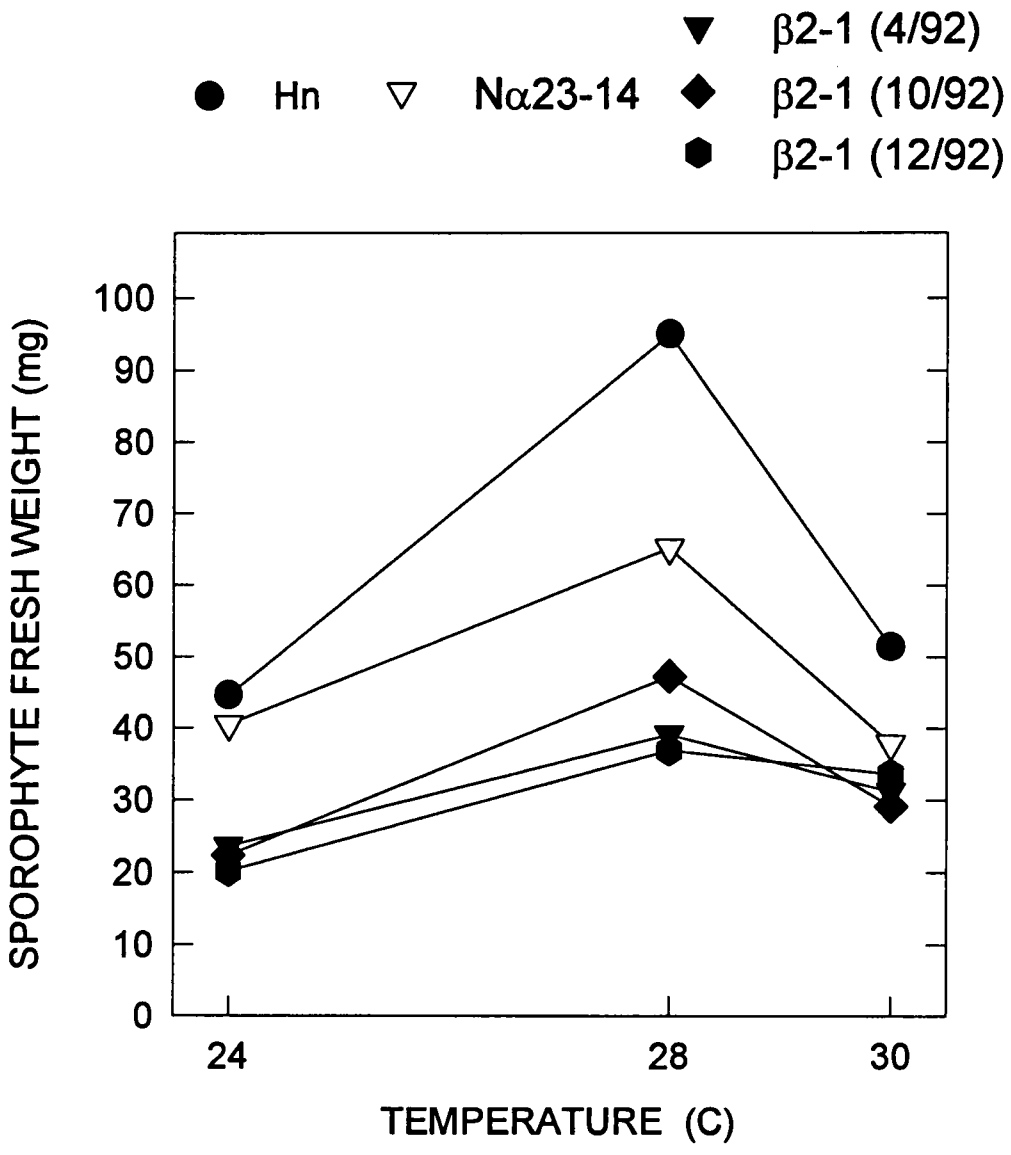

Hn
Nα23-14
β2-1 (4/92)
β2-1 (10/92)
β2-1 (12/92)
SPOROPHYTE FRESH WEIGHT (mg)
100
90
80
70
60
50
40
30
20
10
0
24
28
30
TEMPERATURE (C)

Figure 6

Examination of quantitative data obtained from the previous three experiments (data shown in Figures 2, 4 and 6) revealed substantial variation in the fresh and dry weight of the controls (Table 4). The fresh weights of Hn and β2-1 differed by as much as 40 mg between experiments 1 and 3, whereas the fresh weight of Nα23-14 was reduced by almost half (60 mg) between experiments 1 and 2. The variation in salinity tolerance responses and in fresh and dry weights between experiments in the absence of stress suggested that sources of variation during the *in vitro* culture were present and unaccounted for. Therefore, investigations into the general culture of sporophytes were made.

Culture Improvements

Solidifying agents

The initial selections of the putative tolerant sporophytes (Nα23-14-β1 through Nα23-14-β14) were performed using Hazelton™ agar. However, midway through the establishment of homozygous lines the laboratory underwent a change from Hazelton™ to Difco Bacto™ agar. In an effort to determine the source of variation in fresh weight of the controls (data shown in Table 4) the effects of agar brand and agar washing on sporophyte growth and salinity tolerance of Hn, Nα23-14, β2-1 and β3-10 were investigated. β3-10, another of the M3 generation selections was included because data obtained from the original screen of M3 sporophytes showed

Table 4. Sporophyte fresh and dry weight cultured on agar-solidified (Difco-Bacto™) Parker/Thompson nutrient media at 18-21 DFT of Hn, Nα23-14 and β2-1 (collection date 4/92). Data derived from results shown in Figure 2 (Experiment 1), Figure 4 (Experiment 2) and Figure 6 (Experiment 3). Numbers represent means of 20 sporophytes.

Experiment	Hn		Nα23-14		β2-1	
	Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)
#1	135.9	12.3	114.3	6.3	80.4	5.1
#2	115.7	7.8	58.8	5.4	52.0	4.8
#3	95.0	5.2	65.2	4.7	39.1	2.9

that β3-10 exhibited ~50% NaCl tolerance on Hazelton™ agar and ≤1% on Difco Bacto™ agar (data shown in Table 2). The solidifying agents used included: a) unwashed Phytagel™ (U-Phy), an agar substitute produced from a bacterial substrate composed of glucuronic acid, rhamnose and glucose; b) washed Difco Bacto™ agar (W-Bac); c) unwashed Hazelton™ agar (U-H); d) unwashed Difco Bacto™ agar (U-Bac) and e) washed Hazelton™ agar (W-H).

Comparison of the fresh weight of the 0mM controls on different solidifying agents shows decreasing growth of Hn in the following order: U-Phy, W-Bac, U-H, U-Bac, W-H (Figure 7). However, mean separation showed these differences were not statistically significant (.05 level of probability). Growth of Nα23-14 was significantly increased on U-Phy, W-Bac and W-H when compared to other treatments. Growth of β2-1 and β3-10 was significantly increased on W-Bac when compared to U-Bac and U-H, and U-Bac, respectively. Although fresh weight data indicated slightly better growth of Hn on U-Phy, dry weight data showed this treatment was no different from the others (Figure 7). Mean separation of whole plant dry weight of Nα23-14, β2-1 and β3-10 resulted in no treatments being significantly different.

Also notable in the fresh and dry weight data is the large standard error associated with Hn. Upon reviewing the experimental setup the only identifiable source of variation was the unintentional use of different colored culture trays, specifically grey and black. When strains were separated

Figure 7. Sporophyte fresh and dry weight at 18 DFT of Hn, Nα23-14, β2-1 and β3-10 cultured on Parker/Thompson nutrient media solidified with a variety of agents. Solidifying agents were: Unwashed Phytagel (U-Phy), washed Difco Bacto agar (W-Bac), unwashed Hazelton agar (U-H), unwashed Difco Bacto agar (U-Bac) and washed Hazelton agar (W-H). Bars represent means ± standard error of 20 sporophytes.

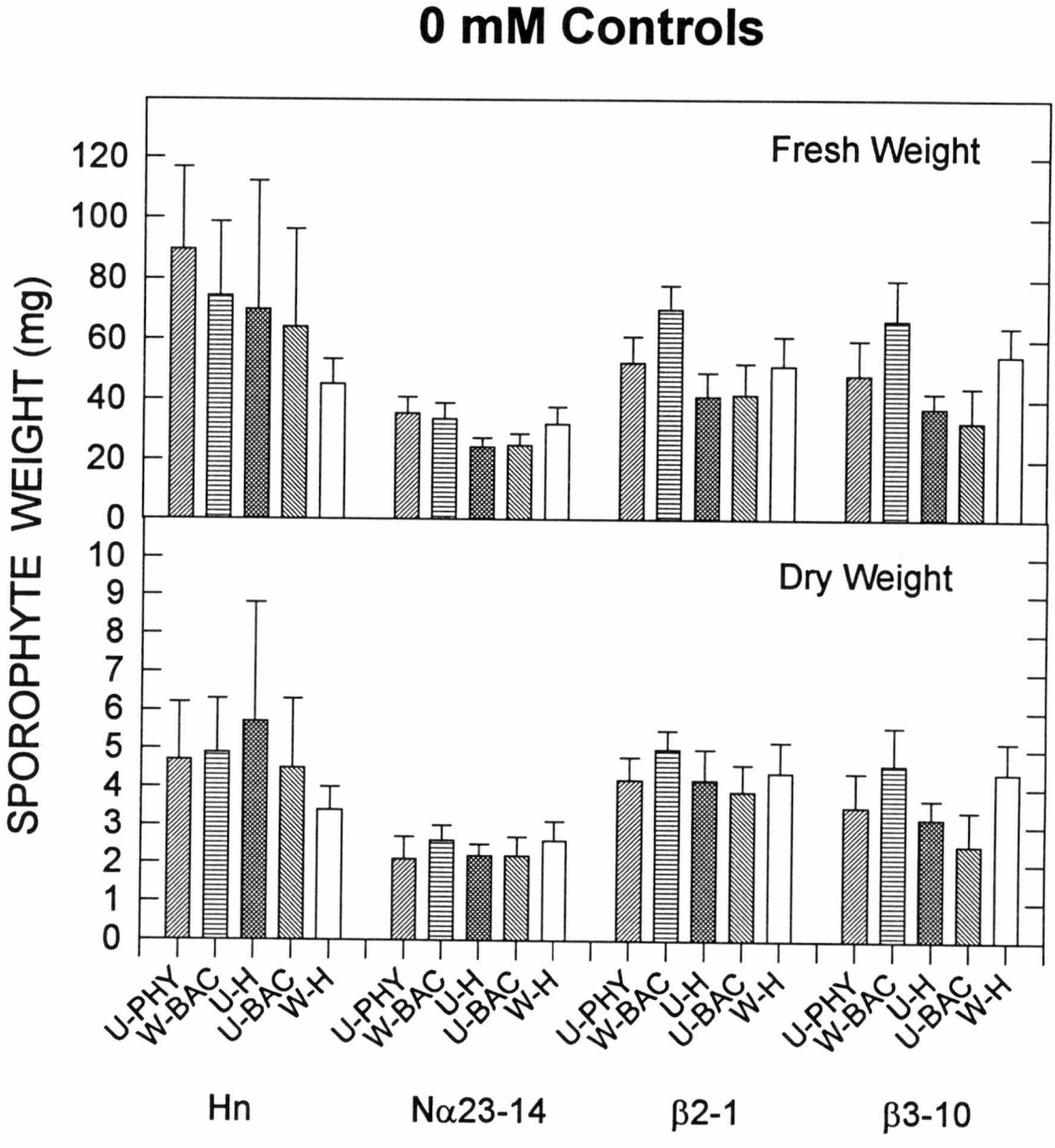

0 mM Controls
Fresh Weight
Dry Weight
SPOROPHYTE WEIGHT (mg)
120
100
80
60
40
20
0
10
9
8
7
6
5
4
3
2
1
0
U-PHY
W-BAC
U-H
U-BAC
W-H
Hn
Nα23-14
β2-1
β3-10

Figure 7

among the culture trays some were placed in grey and black trays (Hn and β3-10) and some only in black trays (Nα23-14 and β2-1). Therefore, there are no data for the effect that tray color differences may have had on Nα23-14 and β2-1. Separation of the data obtained from the different colored trays revealed a difference of at least 50% in fresh weight for Hn sporophytes in grey vs. black trays (Figure 8). β3-10 sporophytes were also affected but to a lesser degree. Statistical analysis showed significant tray*dish interactions for Hn and β3-10 ($P<.05$). Mean separation with the effect of tray (strain*treatment) interactions removed resulted in W-Bac and U-Phy being significantly better for root growth and W-Bac being significantly better for shoot and whole plant growth when compared to the other treatments (Table 5).

Salinity tolerance of β2-1 on 65 mM NaCl appears to be increased with the U-H and U-Bac treatments when compared to the other treatments (Table 6). These differences are less pronounced on 80 mM NaCl. Also, any clear distinction between Nα23-14 and β2-1 on 80 mM NaCl is once again difficult to make. Tolerance of β3-10 on 65 mM NaCl appears to be increased by the U-Phy, W-Bac and U-H treatments. On 80 mM NaCl tolerance appears to be increased by the U-H treatment.

Comparison of the data in Tables 5 and 6 shows U-Phy and W-Bac supported the best growth of the 0mM controls while U-H apparently increased salinity tolerance of β2-1 and β3-10 when compared to other

Figure 8. Sporophyte fresh weight at 18 DFT of Hn and β3-10 cultured on Parker/Thompson nutrient media solidified with a variety of agents in grey and black trays. Solidifying agents were: Unwashed Phytagel (U-Phy); washed Difco Bacto agar (W-BAC); unwashed Hazelton agar (U-H); unwashed Difco Bacto agar (U-BAC) and washed Hazelton agar (W-H). Bars represent means ± standar error of 20 sporophytes.

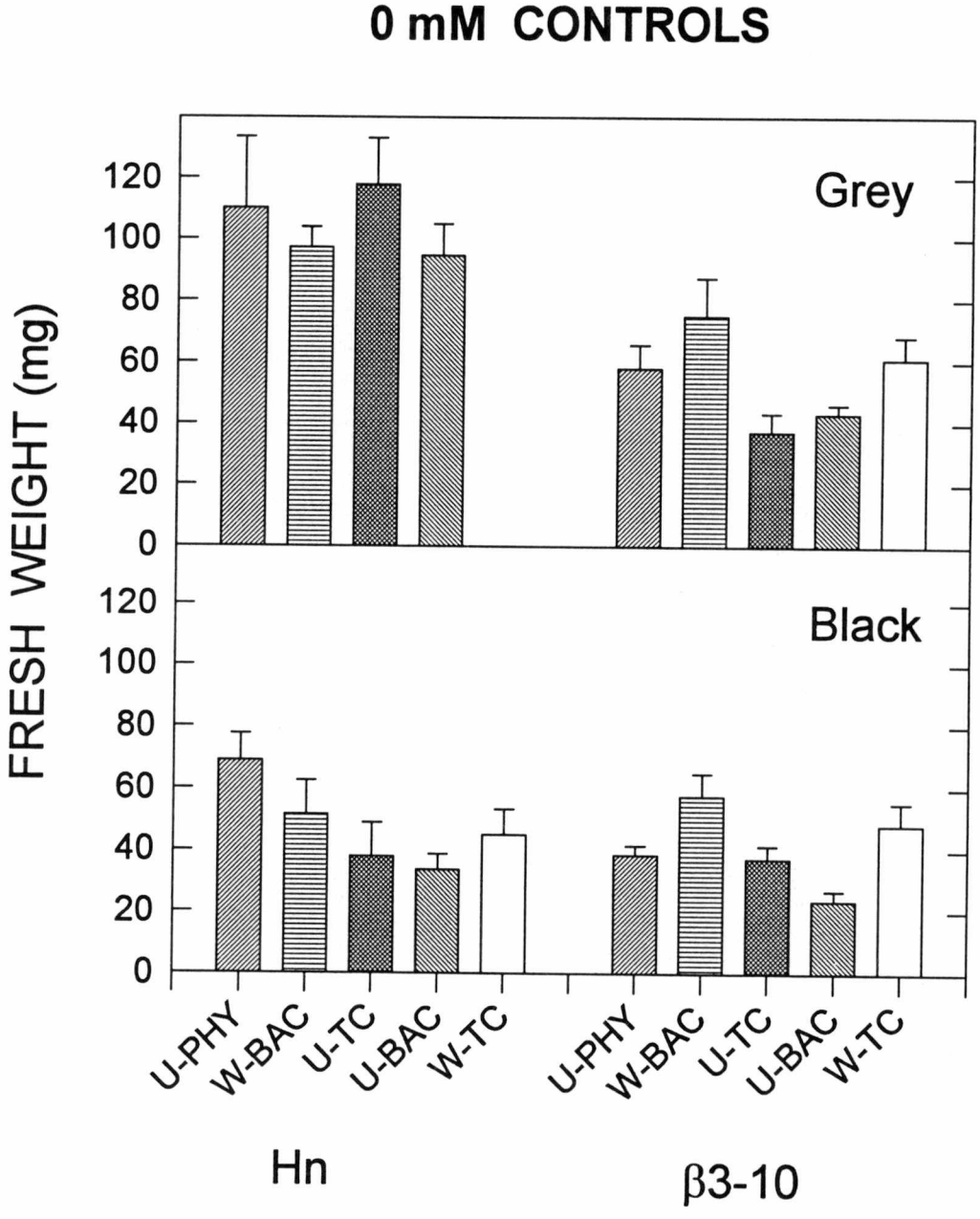

0 mM CONTROLS
FRESH WEIGHT (mg)
Grey
Black
120
100
80
60
40
20
0
U-PHY
W-BAC
U-TC
U-BAC
W-TC
Hn
β3-10

Figure 8

Table 5. Sporophyte shoot, root and whole plant fresh weight at 18 DFT cultured on Parker/Thompson nutrient media solidified with a variety of agents. Solidifying agents were: Unwashed Phytagel™ (U-Phy); washed Difco Bacto™ agar (U-Bac); unwashed Difco Bacto™ agar (U-Bac); washed Hazelton™ agar (W-H) and unwashed Hazelton™ agar (U-H). Means represent 15-20 measurements with effects of strain*treatment and tray(strain*treatment) interactions removed.

Solidifying Agent	Sporophyte Fresh Weight		
	Shoot	Root	Whole
U-Phy	27.6 B[a]	28.9 A	56.3 AB
W-Bac	34.8 A	26.2 A	61.0 A
U-Bac	28.7 AB	12.4 B	40.8 C
W-H	32.4 AB	13.4 B	45.6 ABC
U-H	31.4 AB	10.0 B	41.3 BC

[a]Treatment means within a column followed by the same letter are not significantly different at the 0.05 level of probability.

Table 6. Percent tolerance at 18 DFT of Hn, Nα23-14, β2-1 and β3-10 cultured on Parker/Thompson nutrient media solidified with a variety of agents and supplemented with 65 and 80 mM NaCl. Solidifying agents were: Unwashed Phytagel™ (U-Phy), unwashed Difco Bacto™ agar (U-Bac), washed Difco Bacto™ agar (W-Bac), unwashed Hazelton™ agar (U-H) and washed Hazelton™ agar (W-H).

Strain	Solidifying Agent	NaCl Concentraion	
		65mM	80mM
		% Tolerant	% Tolerant
Hn	U-PHY	0	0
	U-BAC	0	0
	W-BAC	0	0
	U-H	0	0
	W-H	0	0
Nα23-14	U-PHY	0	0
	U-BAC	0	0
	W-BAC	0	0
	U-H	10	5
	W-H	0	0
β2-1	U-PHY	5	0
	U-BAC	15	10
	W-BAC	0	0
	U-H	25	10
	W-H	5	0
β3-10	U-PHY	30	5
	U-BAC	5	0
	W-BAC	25	0
	U-H	40	30
	W-H	0	5

Table 6

treatments. Because of the variation observed due to tray color, the selection of a solidifying agent to use for the remainder of this project was based upon that which supported the best growth of the 0mM controls and not that which may have increased salinity tolerance. Therefore, washed Difco Bacto™ was selected.

Light Intensity/Background color

Light intensity variations found in the culture room had a general trend of higher intensities ($\sim 100 \mu mol\ m^{-2}s^{-1}$) towards the center of benches and lower intensities ($\sim 70 \mu mol\ m^{-2}s^{-1}$) towards the ends. If the growth differences observed in the solidifying agent experiment (see above) were due to light sensitivity one would expect that all cultures directly under the highest intensities would have the same growth regardless of which tray they were in. However, this was not the case. The differences observed were clearly separated by individual tray color. In order to investigate the effect of tray color differences and light intensity on sporophyte growth, four different tray backgrounds were used: black, grey, white and foil. Light intensities ranging from 67 to $120 \mu mol\ m^{-2}s^{-1}$ were tested. In addition, temperatures of each treatment area were measured. There was a general correlation between light intensity and temperature (Figure 9).

Although gametophytes were not of principal interest in this study, there were some notable effects of temperature on their development. Sexual

Figure 9. Correlation between light intensity and temperature. Measurements were taken at various locations in the culture room ($r^2 = 0.694$).

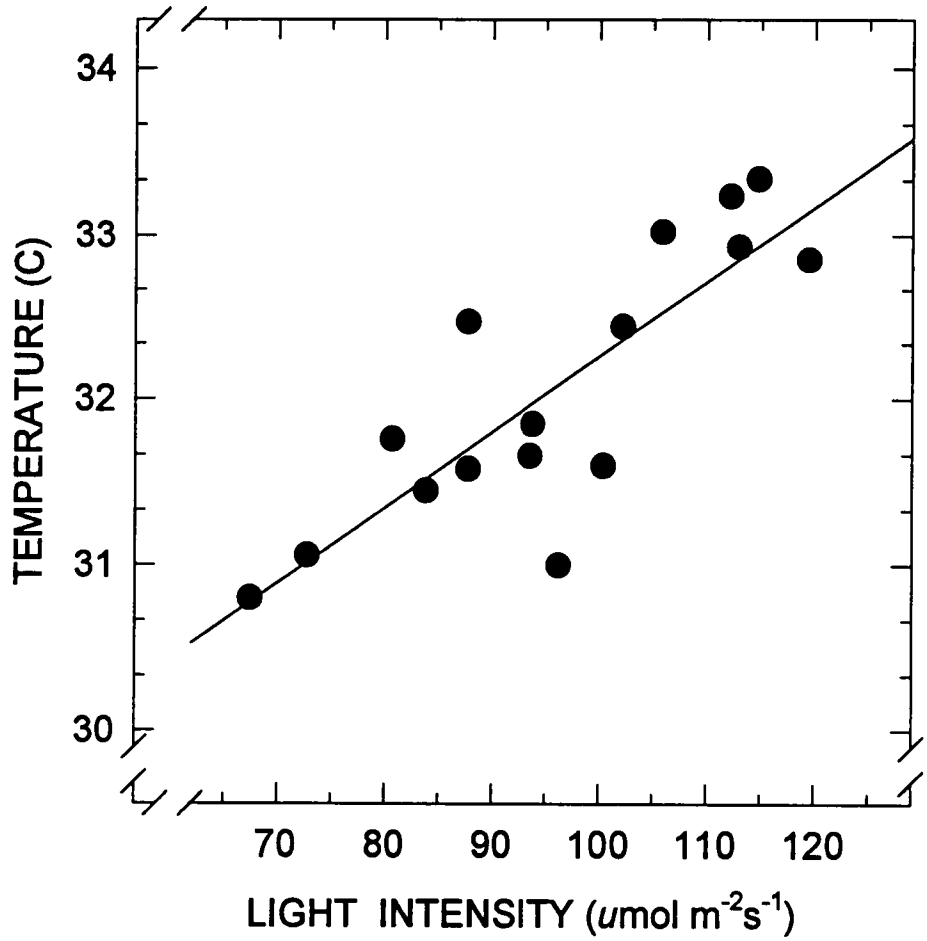

34
33
32
31
30
TEMPERATURE (C)
70
80
90
100
110
120
LIGHT INTENSITY (umol m-2s-1)

Figure 9

development of gametophytes was not effected by different light intensities; all treatments, had ca. 3 archegonia at 12 DFS. In most cases, however, the higher light intensities ($>100\mu$mol $m^{-2}s^{-1}$), did result in reduced gametophyte growth and inhibition of embryo development when compared to lower light intensities. For example, at 8 days from flooding (DFF) embryos in these treatments were comparable to those at 3-4 DFF in the lower intensities. Additional data concerning gametophyte development under various treatments are presented in the Appendix.

In most cases sporophyte growth was severely affected in those cultures subjected to highest light intensities ($>100\mu$mol $m^{-2}s^{-1}$) (Figure 10). Temperature measurements of each treatment show better the differences in sporophyte growth (Figure 11). From these data it was clear that high temperatures, $>32°C$ are inhibitory to sporophyte growth when compared to temperatures $<32°C$.

Mean separation of fresh weight indicated no significant differences among the four backgrounds in the two lower intensity trays. However, there were significant tray differences ($P<.05$) for whole plant and root growth (Figure 12). Unlike the solidifying agent experiment, here there were no other identifiable sources of variation. Although there were no statistical differences among backgrounds, the white and foil backgrounds supported better root growth. Because there was less variation with the white background, it was selected for use in the remainder of this project.

Figure 10. Sporophyte fresh weight at 18 DFT of Hn cultured on agar-solidified (washed Difco-Bacto™) Parker/Thompson nutrient media under different light intensities. Each symbol represents the mean of ten measurements ± standard error from each of four blocks (two lower light intensity blocks and two higher light intensity blocks)(r^2 = 0.462).

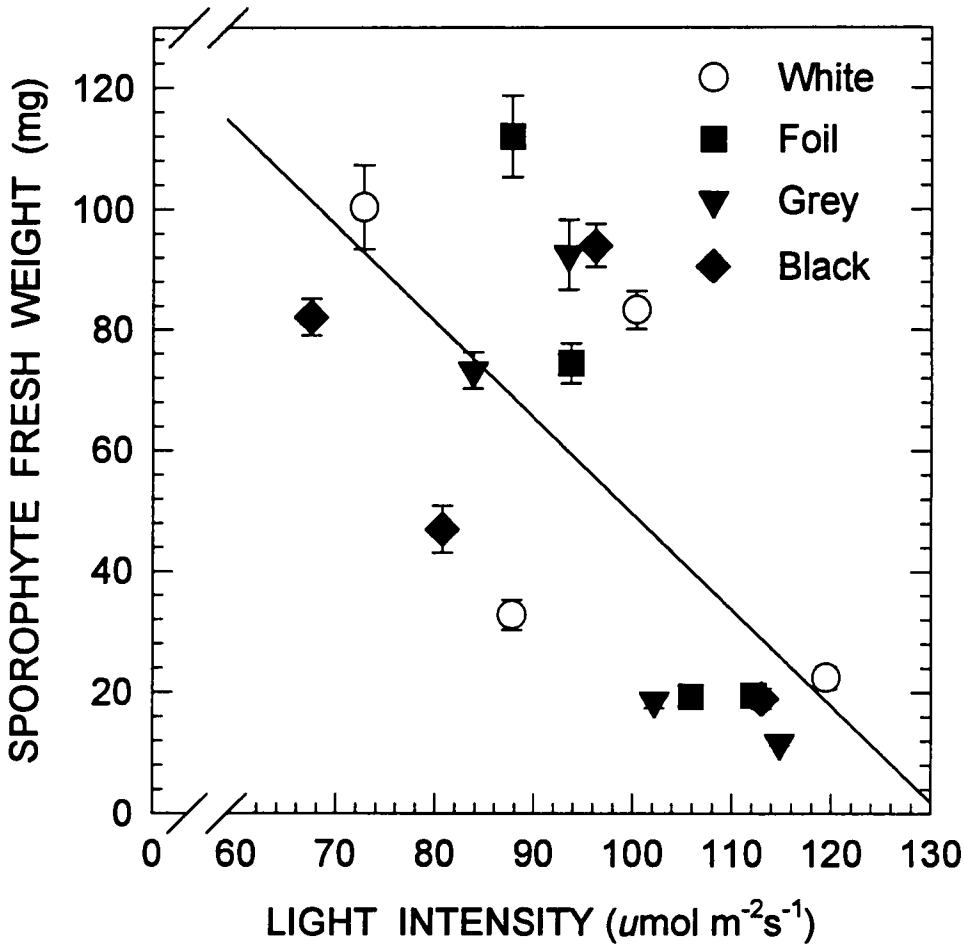

SPOROPHYTE FRESH WEIGHT (mg)
120
100
80
60
40
20
0
0
60
70
80
90
100
110
120
130
LIGHT INTENSITY (umol m-2s-1)
White
Foil
Grey
Black

Figure 10

Figure 11. Sporophyte fresh weight at 18 DFT of Hn cultured on agar-solidified (washed Difco-Bacto™) Parker/Thompson nutrient media under different temperatures. Each symbol represents the mean of ten measurements ± standard error from each of four blocks (two lower light intensity blocks and two higher light intensity blocks)($r^2 = 0.804$).

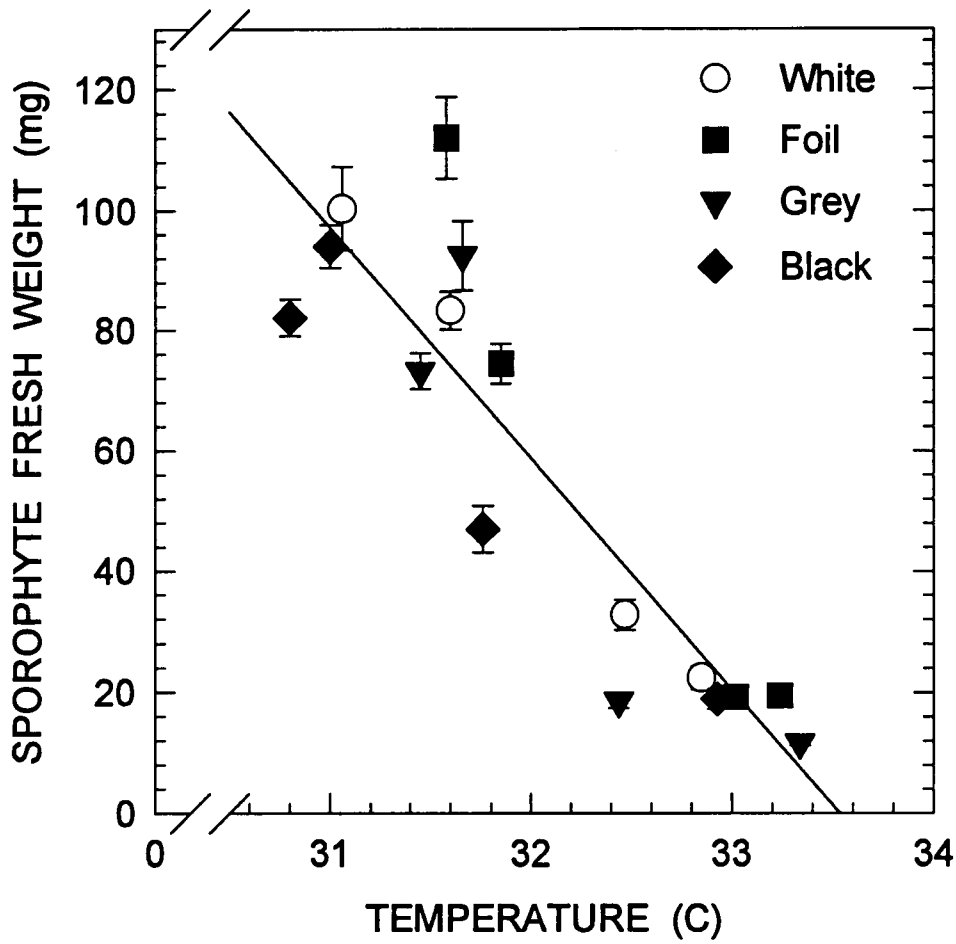

SPOROPHYTE FRESH WEIGHT (mg)
120
100
80
60
40
20
0
0
31
32
33
34
TEMPERATURE (C)
White
Foil
Grey
Black

Figure 11

Figure 12. Sporophyte fresh weight at 18 DFT of Hn cultured on agar-solidified (washed Difco-Bacto™) Parker/Thompson nutrient media under four different tray backgrounds. Data is separated into the two lower light intensity blocks used in this experiment (Trays 1 and 2). Each symbol represents the mean of ten measurements ± standard error.

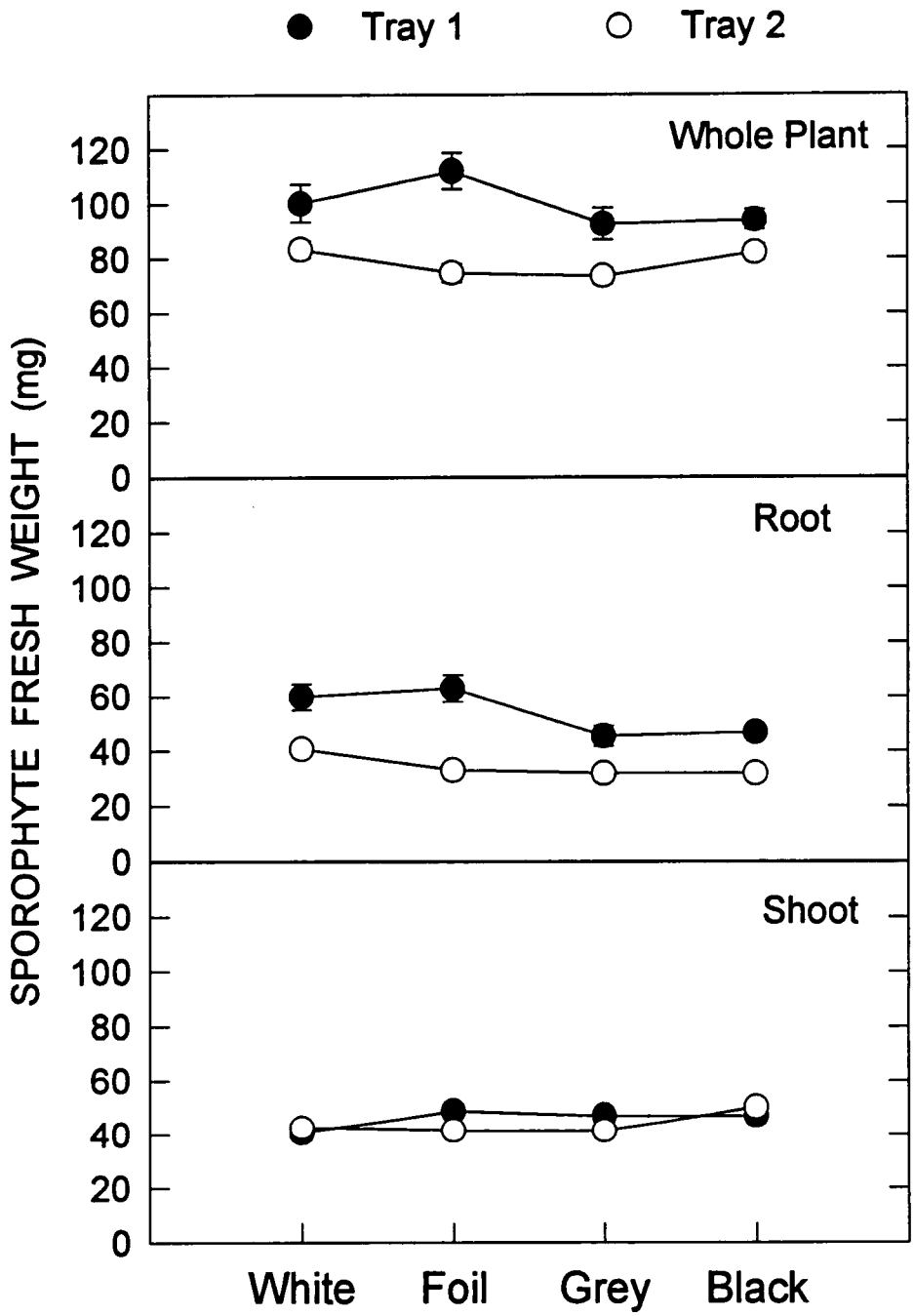

Tray 1
Tray 2
Whole Plant
Root
Shoot
SPOROPHYTE FRESH WEIGHT (mg)
0
20
40
60
80
100
120
White
Foil
Grey
Black

Figure 12

Temperature

In an effort to elucidate the cause of this continued tray to tray variation, the effect of temperature on sporophyte growth was also investigated. Temperatures were monitored within culture trays at various locations in the culture room and a narrow range of 5°C was found. Mean separation indicated the temperature of 27.6°C to be significantly better than other treatments (Table 7). The culture room temperature was lowered in order to acquire an internal tray temperature of ~27°C. However, in subsequent experiments it was noticed that this temperature reduced spore germination rates. Therefore, internal tray temperature was increased and maintained between 28 and 29°C for the remainder of this project. It is important to note that this experiment was not replicated. Therefore, the differences observed may not be associated with temperature, but may also be the result of some other source of variation during the *in vitro* culture of sporophytes.

Nutrient media

There are no published comparative studies involving medium for sporophyte culture of *Ceratopteris*. The Parker/Thompson salts currently used in gametophyte culture will not support long-term sporophyte growth (Hickok et al.,1995). A survey of the literature reveals the use of a variety of media for *in vitro* culture, including the more well known formulas of

Table 7. Effect of temperature on sporophyte growth at 18 DFT on agar-solidified (washed Difco Bacto™) Parker/Thompson media. Means of 20 measurements.

Culture Temperature (C)	Sporophyte Fresh Weight (mg)		
	Root	Shoot	Whole
26.5	87.9 B[a]	102.2 BC	190.1 B
27.6	112.1 A	122.3 A	234.1 A
28.5	72.7 CD	102.9 B	175.2 C
29.6	77.3 C	101.2 BC	178.6 BC
30.3	65.3 D	95.5 C	160.3 D

[a]Treatment means within a column followed by the same letter are not significantly different at the 0.05 level of probability.

Murashige and Skoog, Knop's, Knudson's and Hoagland's. In an attempt to achieve more vigorous sporophyte growth several selected media formulations were tested.

Sporophytes on Hoaglands media had the best overall growth (Figure 13 and Table 8). This seemed to be mostly influenced by the increased root growth. Knop's, White's and Lindemann were restrictive to sporophyte growth. Other treatments were comparable to each other. There were several noticeable visual differences between treatments. DKW 1/2X and DKW 1X sporophytes had a callus like growth that developed at the apical meristem and restricted any leaf development. Sporophytes on Lindeman had marginal necrosis on shoots and poor root growth. Knop's and White's media sporophytes had chlorotic shoots. Although quantitatively, growth on Chu is comparable to most other media tested, there was marginal browning of older leaves.

In addition to short term (18 DFT) observation, stock plates used to generate one-leaf stage sporophyte transfers were observed over a period of 3 months to assess the effects of the different media on multispore culture and long term sporophyte growth. Results of exposure to this longer time period differed somewhat from the 18 day treatment time (Figure 14A and B). Lindeman was toxic to long term sporophyte growth, whereas White's, Knop's and Knudson resulted in small and chlorotic sporophytes . Sporophytes on MS 1/2X and MS 1X and Chu had stunted growth with the

Figure 13. Sporophyte whole plant, shoot and root fresh weight at 18 DFT of Hn cultured under a variety of nutrient media solidified with washed Difco Bacto™ agar. Each symbol represents the mean of twenty measurements ± standard error. The horizontal line drawn through each graph indicates the level of the Parker/Thompson nutrient media control.

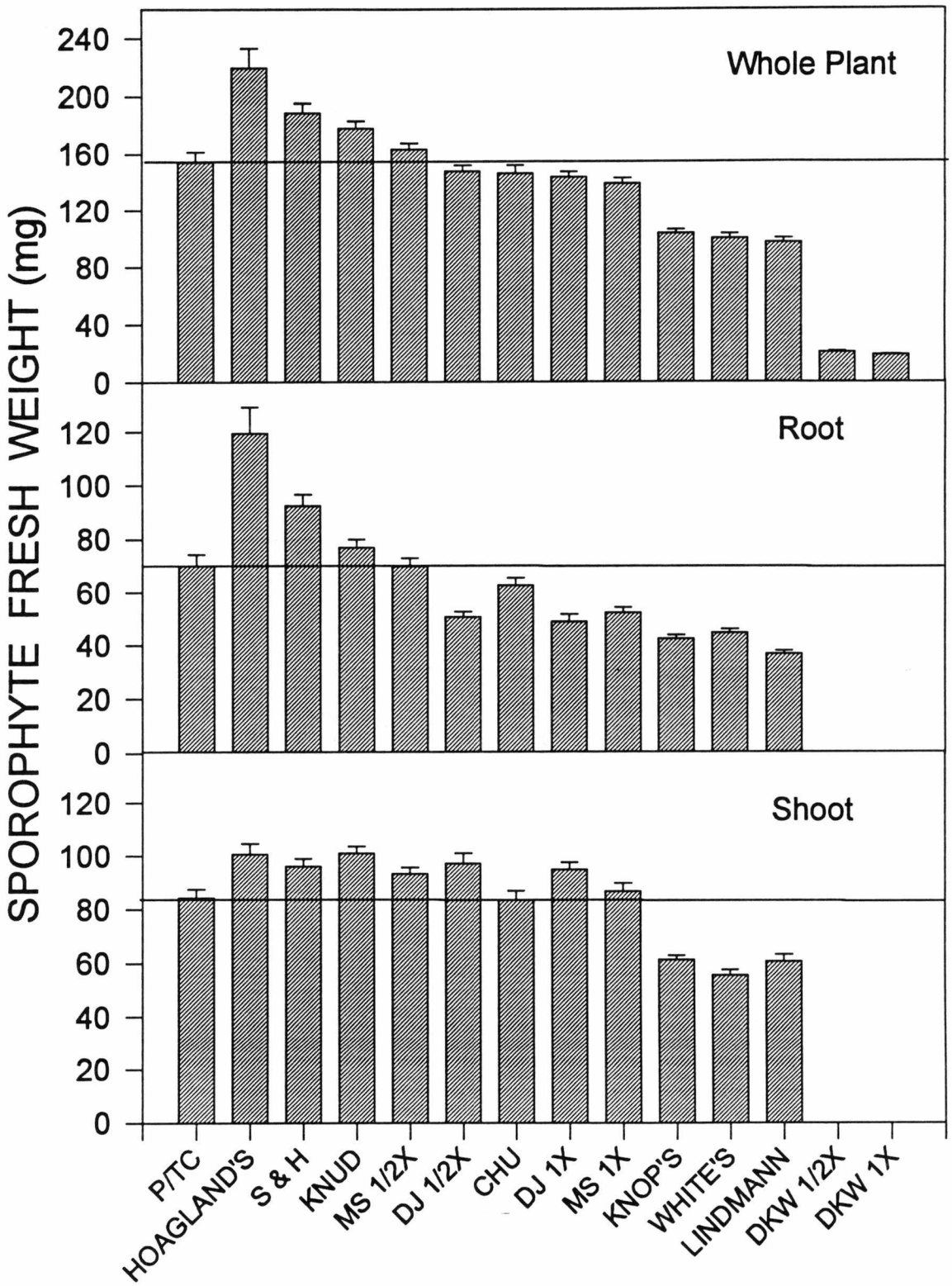

SPOROPHYTE FRESH WEIGHT (mg)
Whole Plant
Root
Shoot
240
200
160
120
80
40
0
120
100
80
60
40
20
0
120
100
80
60
40
20
0
P/TC
HOAGLAND'S
S & H
KNUD
MS 1/2X
DJ 1/2X
CHU
DJ 1X
MS 1X
KNOP'S
WHITE'S
LINDMANN
DKW 1/2X
DKW 1X

Figure 13

Table 8. Sporophyte fresh weight at 18 DFT cultured on a variety of agar-solidified (washed Difco-Bacto™) nutrient media. Means represent twenty measurements.

Nutrient Media	Sporophyte Fresh Weight (mg)		
	Root	Shoot	Whole
Hoagland's	119.3 A[a]	100.6 A	219.9 A
S&H	92.5 B	96.2 A	188.7 B
Knudson's	76.9 C	101.0 A	177.9 BC
MS 1/2X	69.8 CD	93.4 AB	163.2 CD
P/TC	70.1 CD	84.2 C	154.3 ED
DJ 1/2X	50.7 E	97.2 A	147.9 EDF
Chu	62.6 D	83.6 C	146.2 EF
DJ 1X	48.9 EF	95.0 AB	143.8 EF
MS 1X	52.3 E	86.8 BC	135.1 F
Knop's	42.4 FG	58.2 D	103.6 G
White's	45.2 EF	55.6 D	100.2 G
Lindemann	36.9 G	60.6 D	97.4 G

[a]Treatment means within a column followed by the same letter are not significantly different at the 0.05 level of probability.

Figure 14. Sporophyte growth of Hn at (A) 18DFT and (B) 3 months cultured under a variety of nutrient media solidified with washed Difco BactoTM agar.

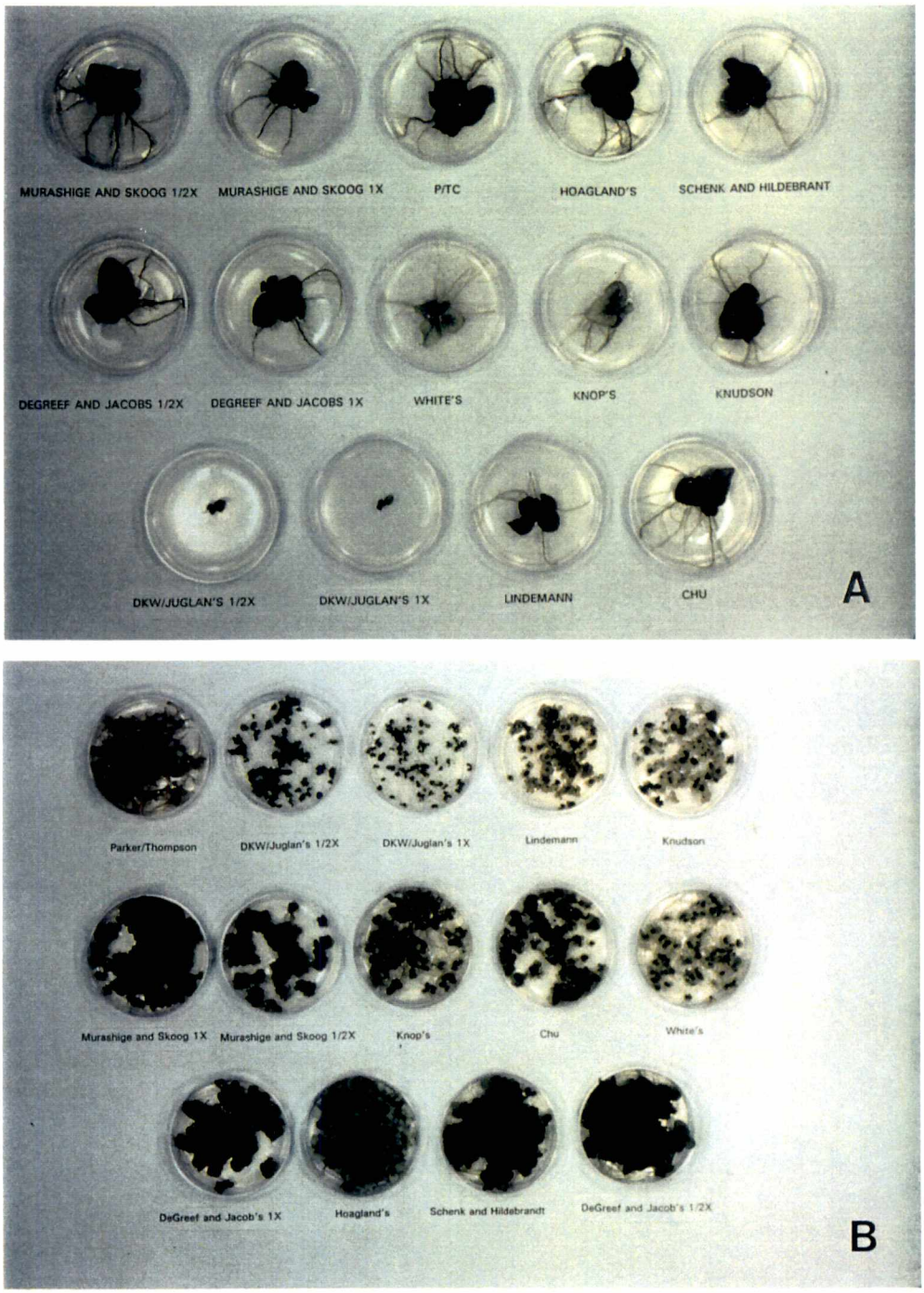

MURASHIGE AND SKOOG 1/2X
MURASHIGE AND SKOOG 1X
P/TC
HOAGLAND'S
SCHENK AND HILDEBRANT
DEGREEF AND JACOBS 1/2X
DEGREEF AND JACOBS 1X
WHITE'S
KNOP'S
KNUDSON
DKW/JUGLAN'S 1/2X
DKW/JUGLAN'S 1X
LINDEMANN
CHU
A
Parker/Thompson
DKW/Juglan's 1/2X
DKW/Juglan's 1X
Lindemann
Knudson
Murashige and Skoog 1X
Murashige and Skoog 1/2X
Knop's
Chu
White's
DeGreef and Jacob's 1X
Hoagland's
Schenk and Hildebrandt
DeGreef and Jacob's 1/2X
B

Figure 14

new growth of shoots being dark green and older growth being chlorotic. Hoagland's supported dense sporophyte growth yet they were a pale green and slightly chlorotic. DJ 1/2X and S & H supported the best growth with DJ 1/2X having extremely vigorous root growth. S & H shoots were slightly paler than DJ 1/2X. There is no data for the effect of DJ 1/2X in long term single sporophyte culture. However, because this treatment supported healthy short and long term growth it was selected for use in general sporophyte culture.

Gibberellic acid

Control groups for the three types of GA_3 preparation had 12-13 leaves and no sporangia present after five weeks of treatment application and six weeks of culture (Figure 15). GA_3 incorporated into the media was toxic at levels of 5.0×10^{-3} M and 10^{-2} M. Although 5.0×10^{-4} M and 10^{-3} M had sporangia present by the 5th leaf, growth was abnormal and severely inhibited. Inhibition of growth was not present with 10^{-4} M, however, sporangial development was not significantly increased relative to the controls. Autoclaved GA_3 applied drop-wise did not significantly effect sporangial development or the heteroblastic leaf sequence. However, 10^{-2} M did have slightly abnormal leaf growth when compared to the control. The two highest concentrations (5.0×10^{-3} M and 10^{-2} M) of filter sterilized GA_3 did significantly affect leaf and sporangial development. The 5.0×10^{-3} M

Figure 15. Sporophyte development after six weeks of culture on agar-solidified (washed Difco-Bacto™) 1/2X DeGreef and Jacobs media and five weeks of GA_3 treatment. (Top) 0 mM controls, (Left) GA_3 incorporated into media and autoclaved and (Right) filter sterilized GA_3 applied by drop-wise application. Two relicates of each treatment are shown. Red dots indicate the presence of sporangia. Scale bars represent 20mm.

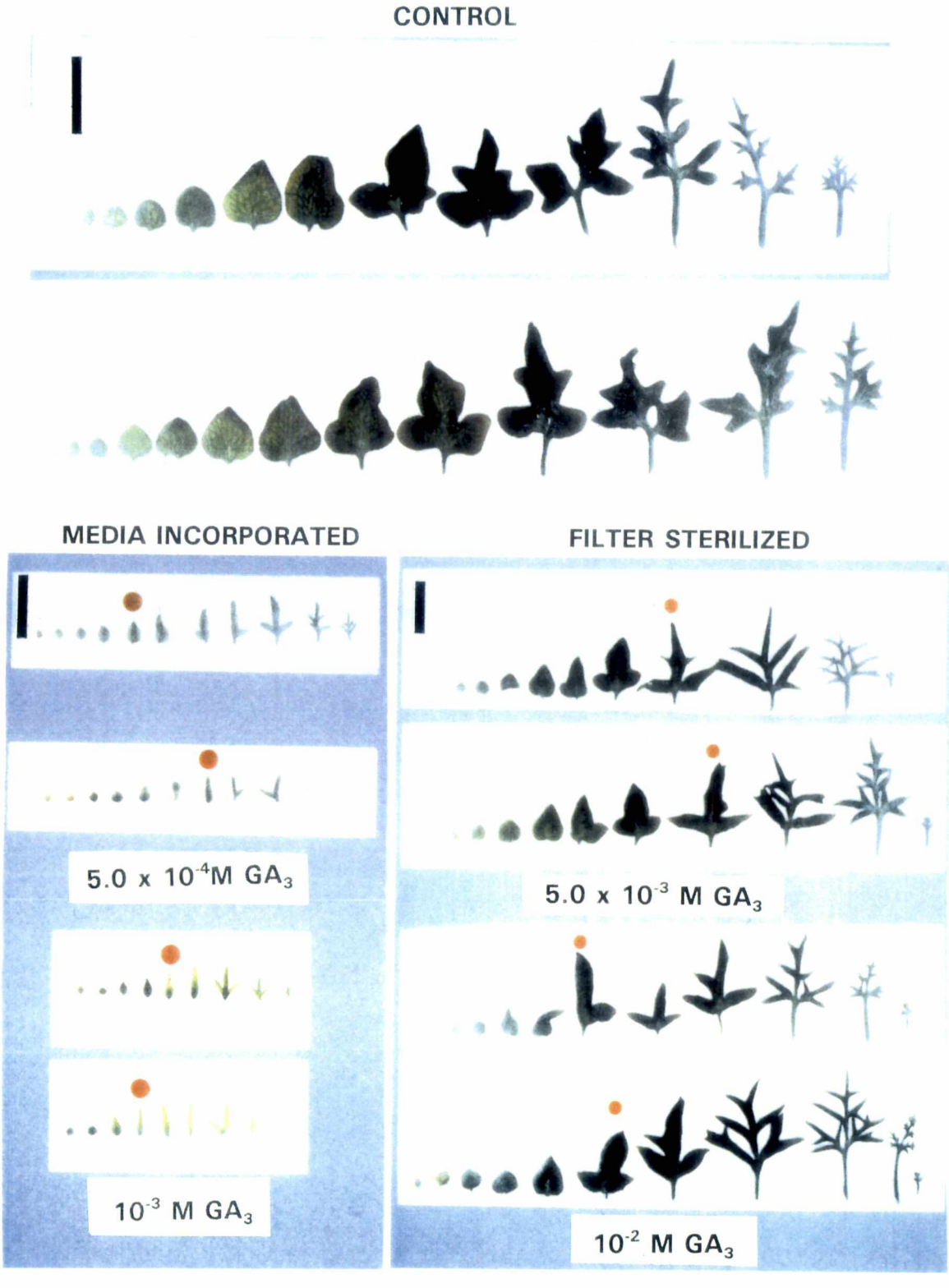

CONTROL
MEDIA INCORPORATED
FILTER STERILIZED
5.0 x 10-4M GA3
10-3 M GA3
5.0 x 10-3 M GA3
10-2 M GA3

Figure 15

and 10^{-2}M treatments had sporangia present by the 9th and 6th leaf respectively. In addition, the heteroblastic leaf sequence appeared to be somewhat accelerated. The remaining concentrations (10^{-4} M, 5.0 x 10^{-4} M and 10^{-3} M) were comparable to the controls.

Comparison of Culture Methods and Salinity Tolerance

The culture improvement investigations, indicated that a tray temperature of 28°C, washed Difco Bacto™ agar, a white background and 1/2X DeGreef and Jacobs media resulted in more vigorous sporophyte growth. These 'new' culture conditions significantly enhanced sporophyte growth for the control groups of Hn, Nα23-14 and β2-1 relative to the 'old' method ($P<.05$)(Figure 16). Hn almost doubled in weight while Nα23-14 almost tripled. Growth of β2-1 was also increased on 0 mM but to a lesser degree. Sporophyte growth on 65 mM and 80 mM NaCl was also significantly increased by the 'new' culture method (Figure 17). Qualitatively, all sporophytes of Hn and Nα23-14 cultured under the 'old' method and supplemented with 65 mM NaCl were necrotic, compared to 50% of β2-1 sporophytes. The 'new' culture method alleviated necrosis on 65 mM NaCl for all sporophytes of β2-1 and for ~80% of Nα23-14. Although quantitatively the increase in fresh weight of all strains on 80 mM NaCl is suggestive of increased tolerance, qualitatively, sporophytes of Hn and Nα23-14 were necrotic (Table 9), while β2-1 sporophytes were chlorotic.

Figure 16. Sporophyte whole plant, shoot and root fresh weight at 18 DFT of Hn, Nα23-14 and β2-1. Two culture methods were employed. The 'Old' method consisted of Parker/Thompson nutrient media solidified with unwashed Difco Bacto™ agar, and a black tray background. The 'New' method consisted of 1/2X De Greef and Jacobs nutrient media solidified with washed Difco Bacto™ agar and a white tray background (Bars represent means of 20 measurements ± standard error).

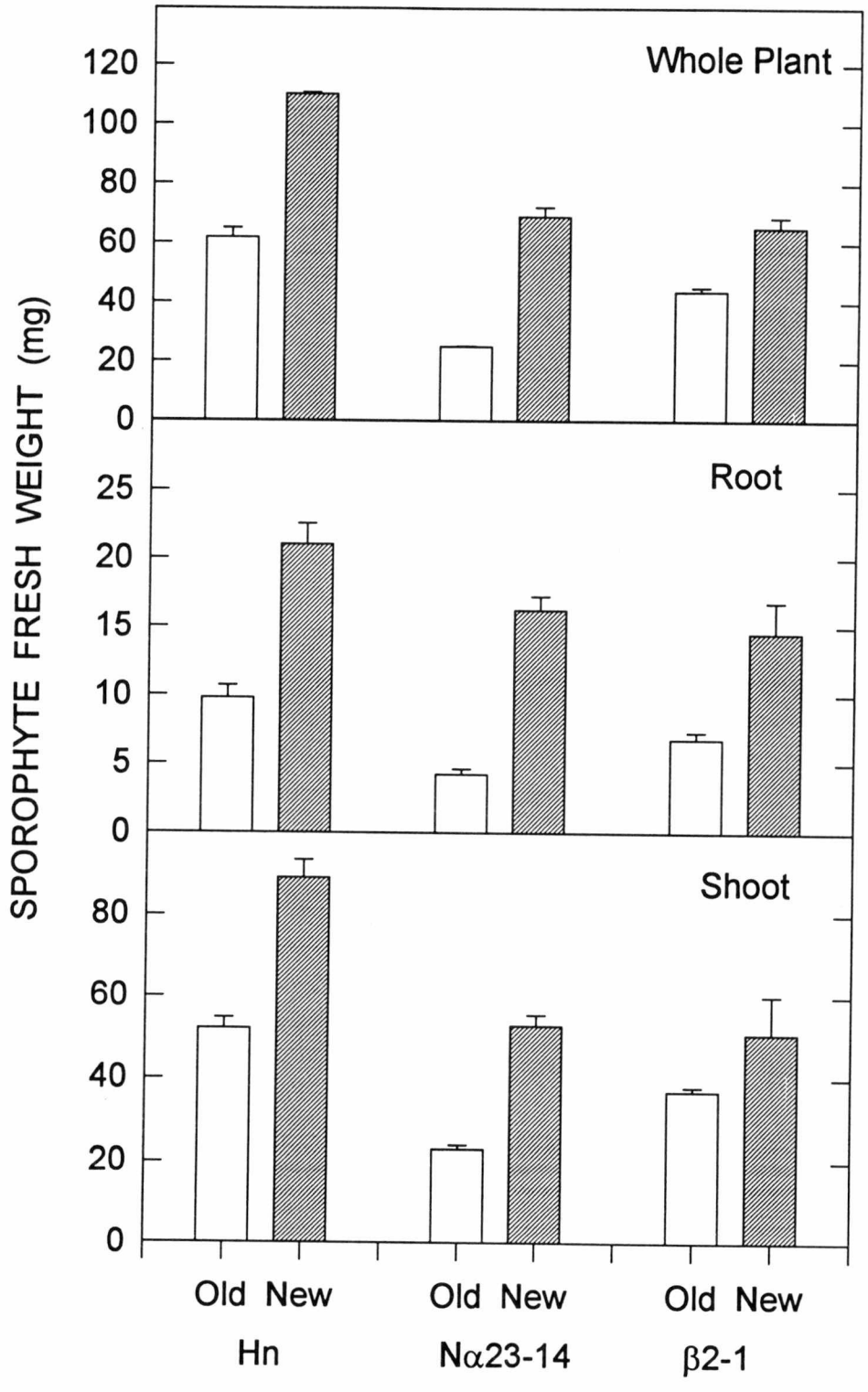

SPOROPHYTE FRESH WEIGHT (mg)
Whole Plant
Root
Shoot
120
100
80
60
40
20
0
25
20
15
10
5
0
80
60
40
20
0
Old New
Hn
Old New
Nα23-14
Old New
β2-1

Figure 16

Figure 17. Sporophyte fresh weight at 18 DFT of Hn, Nα23-14 and β2-1. Two culture methods were employed. The 'Old' method consisted of Parker/Thompson nutrient media solidified with unwashed Difco Bacto™ agar, and a black tray background. The 'New' method consisted of 1/2X De Greef and Jacobs nutrient media solidified with washed Difco Bacto™ agar and a white tray background. Both types of media were supplemented with 65 mM and 80 mM NaCl (Bars represent means of 20 measurements ± standard error)

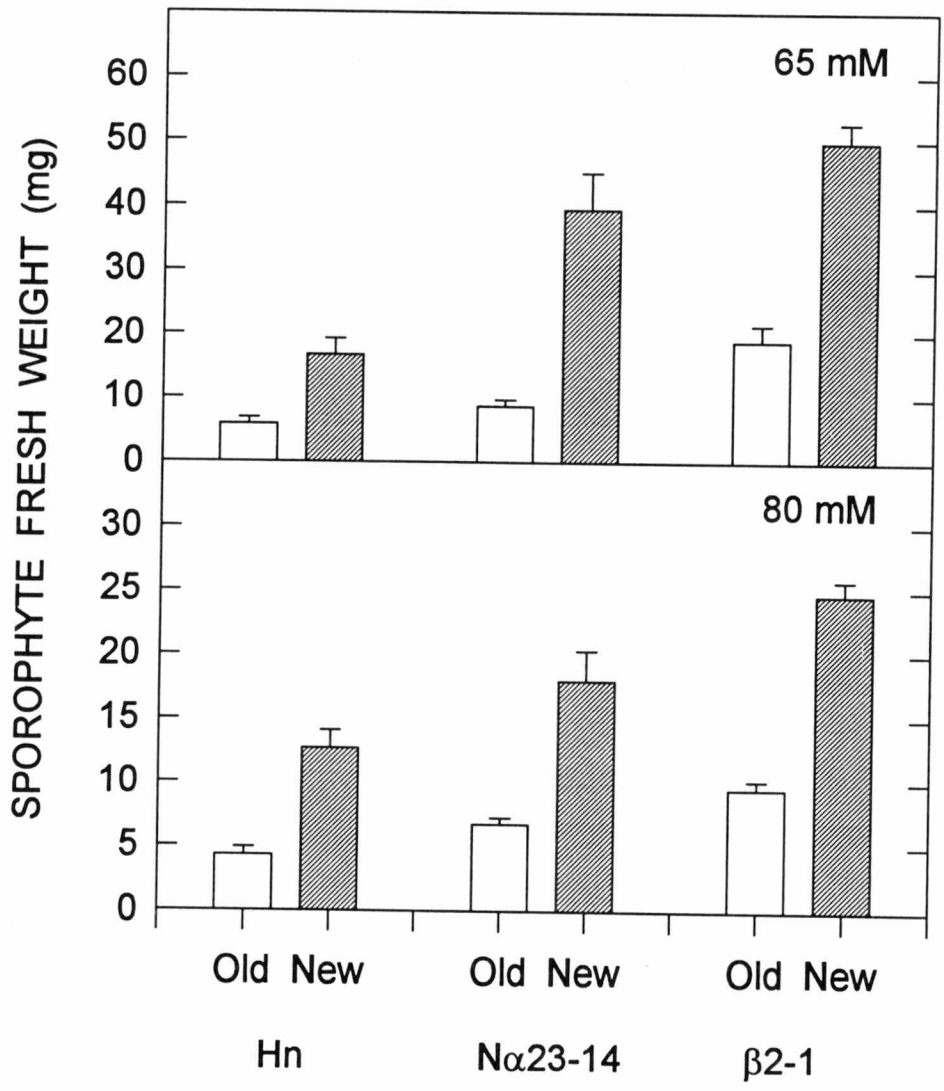

SPOROPHYTE FRESH WEIGHT (mg)
65 mM
60
50
40
30
20
10
0
80 mM
30
25
20
15
10
5
0
Old New
Old New
Old New
Hn
Nα23-14
β2-1

Figure 17

Table 9. Percent tolerance at 18 DFT of Hn, Nα23-14 and β2-1 cultured under two methods ('old' and 'new'). The 'old' method consisted of Parker/Thompson nutrient media solidified with unwashed Difco BactoTM agar, and a black tray background. The 'new' method consisted of 1/2X De Greef and Jacobs nutrient media solidified with washed Difco Bacto™ agar and a white tray background. Both types of media were supplemented with 65 and 80 mM NaCl.

Strain	Culture Method	NaCl Concentration	
		65 mM	80 mM
		% Tolerant	% Tolerant
Hn	old	0	0
	new	0	0
Nα23-14	old	0	0
	new	81	0
β2-1	old	50	0
	new	100	0

IV. DISCUSSION

Selection For Enhanced Sporophytic Tolerance

Selection for enhanced sporophytic tolerance using the Nα23-14 genetic background was successful. Relative to Nα23-14, the fourteen selected lines (Nα23-14-β*) showed an increased percentage of tolerant M2 sporophytes on 80 mM NaCl. In addition, initial dose responses of M3 sporophytes derived from the M2 strain of β2-1 clearly showed β2-1 exhibited a higher level of tolerance relative to Nα23-14 and Hn, suggesting the presence of an additional mutation(s) that as transmitted through the sexual cycle.

Because the generation of M1 sporophytes during the original selection was via intergametophytic selfing, the fourteen selected lines were expected to be heterozygous for any induced mutation(s). Therefore it was likely that the additional mutation(s) were dominant. Given this, models of expected segregation patterns of M1 and M2 sporophytes were considered.

Two simple models are as follows; flow charts of these models are in the Appendix, Figures 18 and 19. Model 1 (Figure 18): if the additional mutation (*stlx*), was a single gene mutation unlinked to *stl2*, and was heterozygous in the M1 sporophyte, 50% of the M2 sporophytes would be expected to exhibit tolerance. Tolerant M2 sporophytes, generated by intragametophytic selfing, would be assumed to be homozygous for both the

stl2 and *stlx* mutations. Therefore, 100% of M3 progeny of these M2 sporophytes, whether generated by intragametophytic or intergametophytic selfing, would be expected to exhibit tolerance. Model 2 (Figure 19): if there were two additional single, unlinked mutations (*stlx* and *stly*), both in heterozygous form and both required for increased salt tolerance, one would expect 25% of M2 sporophytes to exhibit tolerance. These tolerant M2 sporophytes would be assumed to be homozygous for the *stl2*, *stlx* and *stly* mutations. Again, 100% of the M3 sporophytes would be expected to exhibit tolerance. In contrast to expectations from either model, less than 100% of the M3 homozygotes exhibited tolerance (data shown in Table 2). This departure from expectations could possibly be due to a lack of penetrance of the mutation(s). Penetrance is defined as the frequency (percent) with which a (dominant or homozygous recessive) gene manifests itself in the phenotype of the carriers. Penetrance depends on both the genotype and the environment (Rieger et al. 1976). Therefore, according to the results of the initial dose response experiment (data in Figure 2), in which β2-1 exhibited ~70% tolerance, one could say that *stlx* (Model 1), or the combination of *stlx* and *stly* (Model 2), exhibit 70% penetrance. This would alter the expected segregation patterns for both models. In the case of model 1, 35% of the M2 sporophytes and 70% of the M3 sporophytes would be expected to exhibit tolerance. In model 2, 18% of the M2 sporophytes and 70% of the M3 sporophytes would be expected to exhibit

tolerance.

The data show that from the M1 parental line, Nα23-14-β2, 25% of the M2 sporophytes and ~70% of M3 generation sporophytes exhibited tolerance. This pattern does not clearly fit with either of the two models. Furthermore, because of the variability in tolerance associated with subsequent tests of the β2-1 line, it is not possible at this time to discriminate between these two or any other models. It is evident, however, that all comparative tests of β2-1 consistently showed enhanced tolerance relative to the Nα23-14 parental strain. The fact that the increased tolerance was transmitted sexually from the M1 to M2 and M2 to M3 generations is strong evidence of a genetic basis.

Initially, lethal doses allowed for easy differentiation among β2-1, Nα23-14 and Hn. However, much of the qualitative tolerance associated with β2-1 was not evident in the subsequent dose response experiment. Several approaches to discern the reason for the observed variation in qualitative tolerance of the β2-1 strain were made. These included examining different spore collections of β2-1 and monitoring NaCl tolerance responses to controlled variations in culture conditions.

Among five different spore collections tested, the percentage of transfers that exhibited tolerance to 80 mM NaCl and the level of tolerance exhibited by them was not comparable to that previously seen. For example, only three of the five collections exhibited greater than 10% tolerance and of

these none exhibited greater than 27% tolerance. Therefore, the possibility that different spore collections were the cause of the reduced tolerance was considered unlikely.

Temperature sensitivity tests resulted in Hn, Nα23-14 and three spore collections of β2-1 (those exhibiting greater than 10% tolerance in the previous experiment) showing increased tolerance at 24°C and to a lesser degree at 30°C when compared to the 28°C control. However, this increased tolerance was correlated with decreased growth of each strain at the same temperatures. Therefore, the observed increase in tolerance was most likely the result of the reduced growth of each strain at 24°C and 30°C.

The most common method of solidifying culture media is by the addition of agar. It is known that agar is a rich source of impurities, containing more than 100% of the macroelements introduced in basic medium (Debergh 1983). Different brands of agar may vary in the concentrations of the contaminants they contain and thus could potentially influence the growth of the organism being cultured. Of the greatest concern for studies of salinity (NaCl) responses are the amounts of Na^+ and Cl^- in that the amount added from the agar will influence the final molarity of NaCl in prepared media. In addition, by washing the agar, many of the impurities it contains can be removed. In this study, the effects of several brands of agar or solidifying agents (washed and unwashed) on salinity responses were examined. Of

those agents tested, none significantly effected the level of tolerance expressed by β2-1 on 80 mM NaCl. However, differences were seen with β3-10. Consistent with prior observations, sporophytes on unwashed Hazelton™ agar exhibited a higher percent tolerance on 80 mM NaCl when compared to sporophytes on unwashed Difco Bacto™ agar. It is important to note that poor root growth was associated with the 0 mM controls of β3-10 on Hazelton™ agar when compared to unwashed Difco Bacto™ agar. Therefore, the observed increase in tolerance was possibly the result of the reduced root growth which could limit the amount of Na^+ uptake.

The final comparative study of two culture methods 'new' vs. 'old' on salinity stress resulted in a significant amount of increased quantitative tolerance for β2-1. However, this was also true for Nα23-14 and Hn. In addition, growth of the 0 mM controls of each strain was almost doubled by the 'new' culture method. Therefore, there was a direct relation between increased growth and increased tolerance.

In each of the above experiments β2-1 continued to show a quantitatively higher level of tolerance on 80 mM NaCl when compared to Hn and Nα23-14. Moreover, qualitative differences between β2-1 and Nα23-14 were evident on 65 mM NaCl (data shown in Table 9). However, the qualitative differences previously seen between β2-1 and Nα23-14 on 80 mM were non-evident (data shown in Figure 3).

A single gene mutant exhibiting high levels of salinity tolerance in the

sporophytic stage would be advantageous to elucidating the genetics of salt tolerance. However, because of the inconsistencies associated with tolerance of β2-1, this particular strain would be difficult to pursue. The inability to qualitatively differentiate between Nα23-14 and β2-1, in a manner in which one could visually access sensitivity vs. tolerance, makes any genetic characterization of β2-1 extremely difficult. The genetic characterization of β2-1 would be essential if this strain was to be further investigated. As an alternative approach, it may be advantageous to initially select for tolerance on a higher level of NaCl. If the screening of M1 mutants was performed on 100, 125 or even 150 mM NaCl this could result in more vigorous growth on 80 mM NaCl and perhaps even higher salinity levels and would allow for easier differentiation among strains.

Culture Improvements

Comparison of the responses of the 0 mM controls of Hn, Nα23-14, β2-1 and β3-10 to the various solidifying agents differed among the strains tested. Hn and Nα23-14 had best overall growth (as determined by fresh weight) on Phytagel™. However, when dry weight data were considered, the positive effect observed with Phytagel™ was greatly decreased. This was most likely the result of vitrification or hyperhydric transformation (Debergh 1983). β2-1 and β3-10 had best overall growth on washed Difco Bacto™ agar. Root growth was significantly reduced on unwashed

Hazelton™ and unwashed Difco Bacto™ agars and significantly enhanced on washed Difco Bacto™ and Phytagel™. Upon removal of identifiable sources of variation, W-Bac and U-Phy supported to best growth. Because of the problem of vitrification seen with Phytagel, washed Difco Bacto™ was chosen for use in the general culture of sporophytes.

Although it has been shown that high light intensity (480×10^4 ergs cm^{-2} s^{-1}) inhibits sporophyte development in the heterosporous fern *Marsilea* (Mahlberg and Yarus, 1977), investigations into the effect of light intensity and background color resulted in little correlation between sporophyte growth and direct light intensity for *Ceratopteris*. There was however a general association between high light and high temperatures. Those trays which were raised to increase light intensity had an average temperature of 33°C compared to 31°C of the lower intensity (bench level) trays. Three treatments had light intensities of $\sim 100 \mu mol\ m^{-2}s^{-1}$ (two in the raised trays and one on bench level), however, sporophytes in the raised trays had arrested and irregular development. In addition, although the two lowest light intensities in the raised trays did support the best growth among those treatments, development was slower when compared to the bench trays. The inhibition of sporophyte growth in the higher intensity cultures was most likely due to the higher temperature within those trays. Better root growth of foil and white backgrounds in the lower intensity trays (bench level), may be the result of the high amount of light that is reflected up

through the plate bottom. Higher values of reflected light were consistent with better root growth in the lower trays. Because the white background supported good growth and had the lowest amount of variation between the trays, when compared to the foil background, I selected it as a standard condition for sporophyte culture.

In one study investigating the effect of temperature on sporophyte development, Mahlberg and Yarus (1977) showed that low (15°C) and high temperatures (35°C) inhibited sporophyte formation while optimum development occurred at 25°C. Investigations into the effect of the 5°C range of temperature found in the culture room on sporophyte growth showed a temperature of 27.6°C was significantly better when compared to 26.5°C, 28.5°C, 29.6°C and 30.3°C. However because this temperature decreased spore germination rates culture trays were maintained between 28 and 29°C.

There were several differences observed in sporophyte growth on the various nutrient media tested. Although gametophytes have not been mentioned here (data available in the Appendix), there were interesting differences in the effect of the media on the two life cycle stages. Lindemann, MS 1/2X and 1X supported healthy gametophyte growth. However, sporophytes on Lindemann were necrotic, possibly the result of aluminum toxicity. Sporophytes on MS 1/2X and 1X had reduced growth, chlorotic older leaves and dark green new growth, a symptom of nitrogen

toxicity. Gametophytes on S&H had reduced growth and were chlorotic, yet this media supported healthy sporophyte growth. This difference could possibly be due to the high levels of potassium and nitrate in the media. Callus formation on both gametophytes and sporophytes grown on DKW was most likely the result of one or more of the organic compounds it contains (6-benzylaminopurine (BA), glycine, indole-3-butyric acid, myo-inositol and nicotinic acid). Chlorosis of sporophytes cultured on Whites and Knops media may be attributable to the lack of NH_4^+ in these media. Gametophytes on Whites and Knops also exhibited poor growth. Hoagland's and De Greef and Jacobs media (1/2X) supported healthy gametophyte and sporophyte growth. In multispore cultures observed over ~3 months sporophyte growth on De Greef and Jacobs was vigorous whereas sporophytes on Hoagland's became chlorotic. Because of the healthy growth observed in isolate and multispore cultures, 1/2X De Greef and Jacobs was selected for the general culture of sporophytes. An additional benefit of using DeGreef and Jacobs media is its commercial availability. It is sold in powder form and comes pre-packaged in one liter units.

The comparative study of the two culture methods, 'old' and 'new', 'Old' being that method used prior to this project, and 'new' the method which incorporated all the improvements made during this project, resulted in significant enhancement of overall growth (root and shoot) for all strains (Hn, Nα23-14 and β2-1).

Attempts to complete the life cycle *in vitro* rather than in the greenhouse were unsuccessful. Differences observed between the drop applications of autoclaved vs. filter sterilized GA_3 were most likely attributable to the loss of some activity when autoclaved. 10^{-2}M gave the best results with sporangial development occurring earlier than the controls. However, this concentration is impractical for routine laboratory work due to its high cost. I believe weaker concentrations (less than 10^{-4}) incorporated into the media by filter sterilization may give the desired results. Also, the viability of spores induced by GA_3 should be investigated.

From this project, attempts to improve the general culture of sporophytes were successful. However, several potential sources of variation were found. Although randomized block designs are generally more used in field experimentation, I feel it is important to employ this design when conducting large scale *in vitro* sporophyte experimentation. Possible temperature differences between blocks should be used as covariates when performing statistical analyses. Also, the transfer of one sporophyte to an individual (60 x 15 mm) petri dish rather than the transfer of several sporophytes to large (100 x 15 mm) dishes should be done. Individual transfers will remove the effect of nutrient competition among sporophytes which could potentially influence growth. By using the above design, possible sources of variation can be controlled for and removed from statistical analysis.

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APPENDIX

Table 10. Concentrations of ions in selected plant tissue culture basal salts (Sigma Plant Culture Catalogue, 1993).

mM	P/TC	Whites	Hoagland's	DeGroef and Jacob's (1X)	DeGroef and Jacob's (1/2 X)	Lindemann	DKW/ Juglan's (1X)	DKW/ Juglan's (1/2X)	MS (1X)	MS (1/2X)	Schenk and Hildebrandt	Knop's	Knudson	Chu
NH_4^+	1.6		1.0	6.0	3.0	15.2	17.7	8.9	10.3	5.2	2.6		3.8	7.0
K^+	3.7	1.7	6.0	7.9	4.0	15.1	20.0	10.0	10.7	5.4	24.7	2.1	1.4	30.9
Mg^{+2}	0.48	3.0	2.0	2.0	1.0	0.5	3.0	1.5	1.5	0.8	1.6	0.5	0.5	0.75
Ca^{+2}	0.18	1.3	4.0	2.0	1.0	2.1	9.3	4.7	3.0	1.5	1.4	2.1	2.1	1.1
Na^+	0.20	2.9		2.3	1.2		0.3	.15	0.2	0.1	0.1	0.1 uM	0.1 uM	0.2
NO_3^-	1.6	3.3	14.0	19.8	9.9	4.2	34.4	17.2	19.7	9.9	24.7	5.4	4.2	28.0
PO_4^{-3}	3.7	0.14	1.0	2.1	1.1	1.0	2.0	1.0	1.25	0.6	2.6	0.9	0.7	2.9
SO_4^{-3}	0.59	4.5	2.0	5.2	2.6	8.2	12.3	6.2	1.75	0.8	1.7	0.5	2.4	4.4
Cl	0.036	0.87		12.2	6.1	14.1	2.0	1.0	3.0	1.5	2.7	0.013	0.013	2.3
(μM)														
Al						0.42								
B	30.1	24.0	46.0	172.0	86	16.4	78.0	39	100.0	50.0	81.0	16.2	16.2	76.0
Co				0.01	.005				0.09	0.04	0.42	0.10	0.10	
Cu	2.1	0.04	0.32	0.01	.005	0.76	1.0	0.5	0.10	0.05	0.08	0.045	0.045	
Fe	100		9.0	100.0	50	18.0	122.0	61	100.0	50.0	54.0	30	30	100.0
I		4.5		9.5	4.8	0.6			5.0	2.5	6.0			4.8
Mn	1.5	22.0	9.2	10.0	5.0	0.31	198.0	99	122.0	61.0	59.0	6.32	6.32	19.5
Mo	0.2	0.007	0.11	0.01	.005		1.6	0.8	1.39	0.70	0.41	0.052	0.052	
Ni						0.13	0.02	0.02						
Zn	1.8	10.0	0.76	3.7	1.9	2.0	57.0	28.5	29.9	15.0	3.5	0.087	0.087	5.2

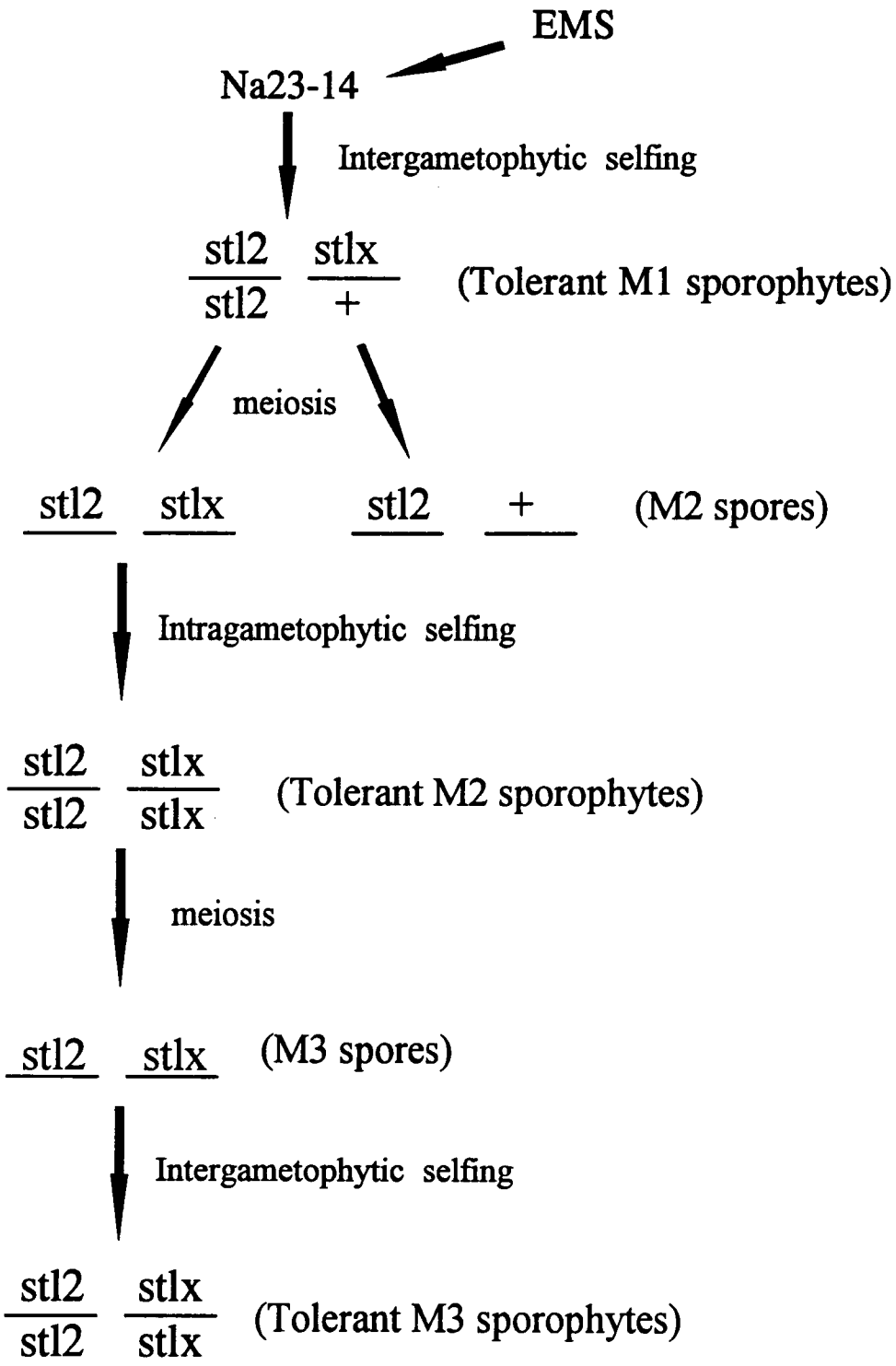


Figure 18. Predicted segregation pattern of a single unlinked mutation (stlx) conferring enhanced NaCl tolerance in sporophytes of *Ceratopteris richardii* containing the *stl2* mutation.

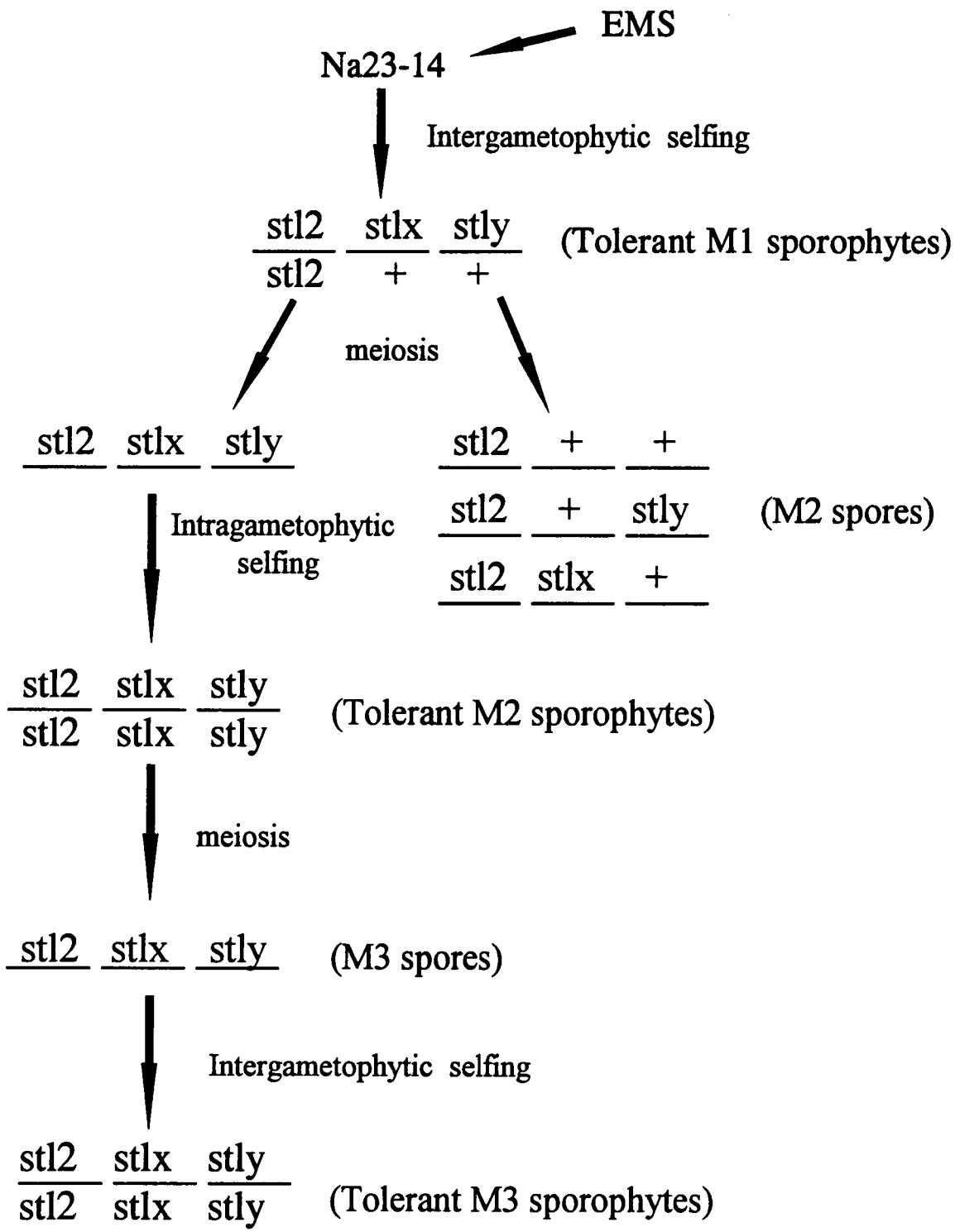


Figure 19. Predicted segregation pattern of two unlinked mutations (stlx and stly) conferring enhanced NaCl tolerance in sporophytes of *Ceratopteris richardii* containing the stl2 mutation.

EFFECT OF TEMPERATURE AND NUTRIENT MEDIA ON GROWTH OF GAMETOPHYTES OF CERATOPTERIS

I. INTRODUCTION

In addition to the culture improvements for sporophytes of *Ceratopteris richardii*, certain aspects of culture conditions were also investigated to asses their effects on gametophytes. Specifically, the effect of temperature on spore germination and gametophyte growth and the effect of various nutrient media on gametophyte growth were investigated.

II. MATERIALS AND METHOD

General Culture

General culture conditions are as described in the main body of the manuscript.

Germination

Spore germination, as identified by an emergent primary rhizoid, was determined from microscopic observation (X25) of 100 spores per 60 x 25 mm petri dish. Initial time reference for timed germination counts was the time spores were inoculated. Replicates of each experiment were run simultaneously and the data combined for analysis.

Growth measurements

Gametophytic growth comparisons were performed by measuring the areas of 10 day old gametophytes. Germinating spores were transferred to treatment petri plates at the primary rhizoid stage. At 10 days from transfer (DFT), gametophytes were removed from treatments and mounted on slides in a fresh 1:4 (v/v) mixture of 0.5% (w/v) acetocarmine and Hoyer's medium (Beeks, 1955). Areas were measured using the computer-interfaced BioQuant™ Image Analysis System.

Temperature

The effect of temperature on germination rates and gametophytic growth were performed on the wild type strain (Hn). Temperatures within culture trays were continuously monitored using temperature probes coupled to a LI-1000 Datalogger from Li-Cor Inc. Treatment temperatures included 26.5 ± .09°C, 27.6 ± .13°C, 28.5 ± .21°C, 29.6 ± .04°C, 30.3 ± .32°C.

Multispore cultures were established and germination rates determined as previously described. To obtain a uniform population for growth studies, multi-spore cultures were germinated at 29.6°C. Germinating spores were transferred at the primary rhizoid stage (3-4 days from sowing) to fresh plates and placed in each temperature treatment (10 transfers per 60 x 15 mm petri dish). Gametophytes were removed from treatment at 10 DFT, mounted on slides and areas measured as previously described. Four

replicates of each temperature were run simultaneously and the data combined for analysis.

Nutrient Media

Spore germination rates and gametophytic growth comparisons of the wild type strain (Hn) on a variety of commercially available media (Appendix, Table 10) were performed . Multispore cultures were established and germination rates determined as previously described. Because no significant differences were seen in spore germination rates on the different media (data not shown), uniform populations were attainable over the range of media. Germinating spores were transferred at the primary rhizoid stage to fresh treatment plates (15 germinating spores per 60 x 15 mm petri dish). Gametophytes were removed from treatment at 10 DFT, mounted on slides and areas measured as previously described. Two replicate plates of each medium were run simultaneously and the data combined for analysis.

III. RESULTS AND DISCUSSION

Temperature

Temperatures monitored at various locations in the culture room revealed a range of 5°C. It was of interest to determine the effect that these differences had on spore germination. At 96 hours from soaking (HFS) 50% of the spores in the two higher temperatures (29.6°C and 30.3°C) had

germinated compared with ~20% of those at 27.6°C, 28.5°C, and less than 10% of those at 26.5°C (Figure 20). From these data it is clear that the lower temperature of 26.5°C significantly decreased spore germination rates whereas the two higher temperatures of 29.6°C and 30.3°C increased spore germination rates.

Measurements of gametophyte growth at the same temperatures resulted in variable responses (Figure 21). Temperatures of 27.6°C and 29.6°C had the best growth with 26.5°C, 28.5°C and 30.3°C having decreasing growth respectively. Because spore germination rates are slower at lower temperatures and gametophyte growth is decreased at 30.3°C while sporophyte growth is decreased at 29.6°C and 30.3°C (data shown in Table 7) , the temperature of 28.5°C was selected for use in the remainder of this project.

Nutrient media

Growth responses on a variety of culture media are represented in Figure 22. DJ 1/2X supported the best growth with MS 1X and 1/2X slightly better than the control of P/TC. Growth was most restricted on S&H, Knop's and DKW 1X. The remaining treatments had no significant effect when compared to the Parker/Thompson control.

There were noticeable visual differences among treatments. DKW 1/2X and 1X had a callus like growth in the meristematic region. Knudson,

Figure 20. Percent germination of Hn cultured under five temperatures. Symbols represent means ± standard error of four replicates for each temperature.

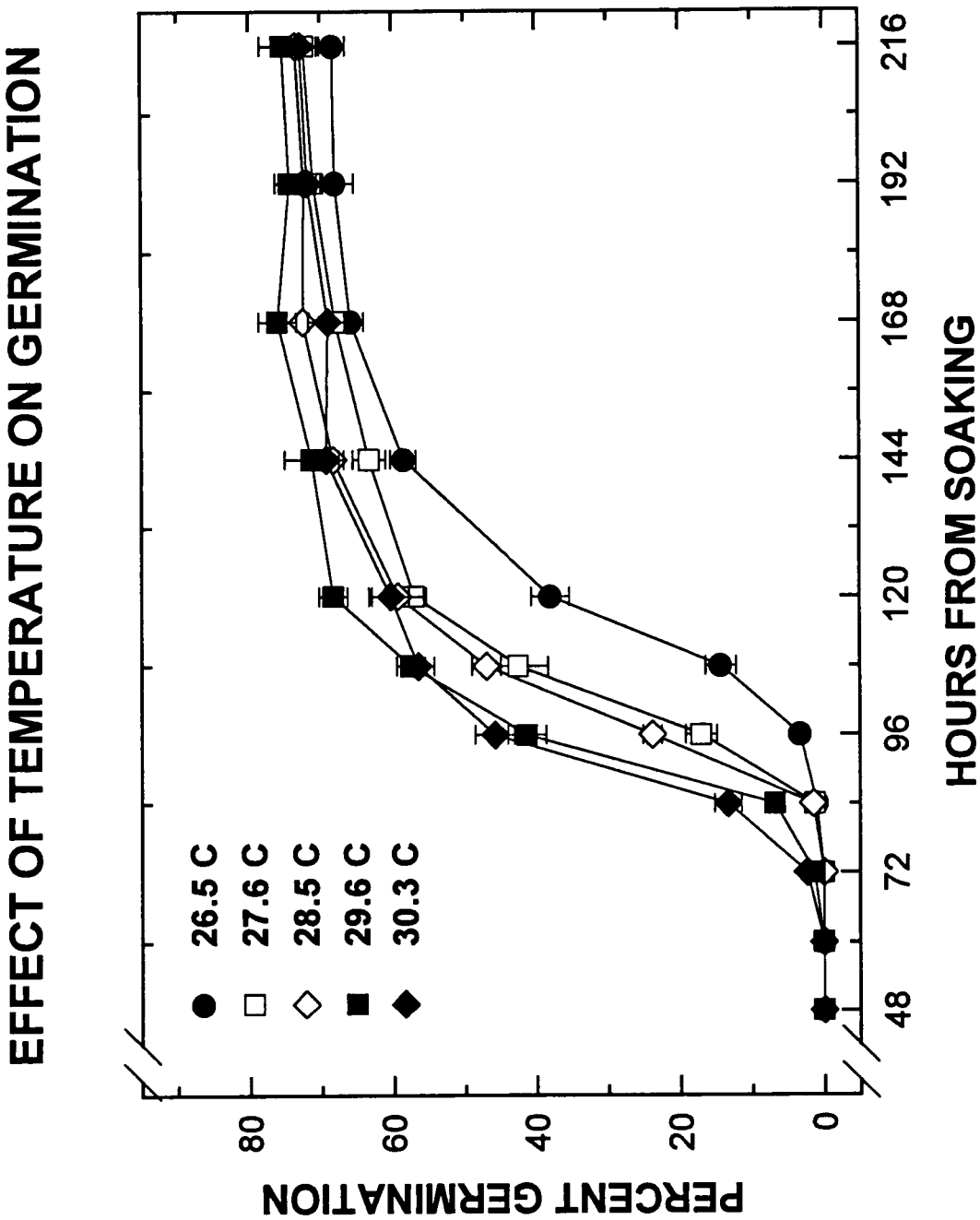

EFFECT OF TEMPERATURE ON GERMINATION
PERCENT GERMINATION
0
20
40
60
80
HOURS FROM SOAKING
48
72
96
120
144
168
192
216
26.5 C
27.6 C
28.5 C
29.6 C
30.3 C

Figure 20

Figure 21. Gametophyte area at 10 DFT of Hn cultured under five different temperatures. Symbols represent means ± standard error of 30-36 measurements.

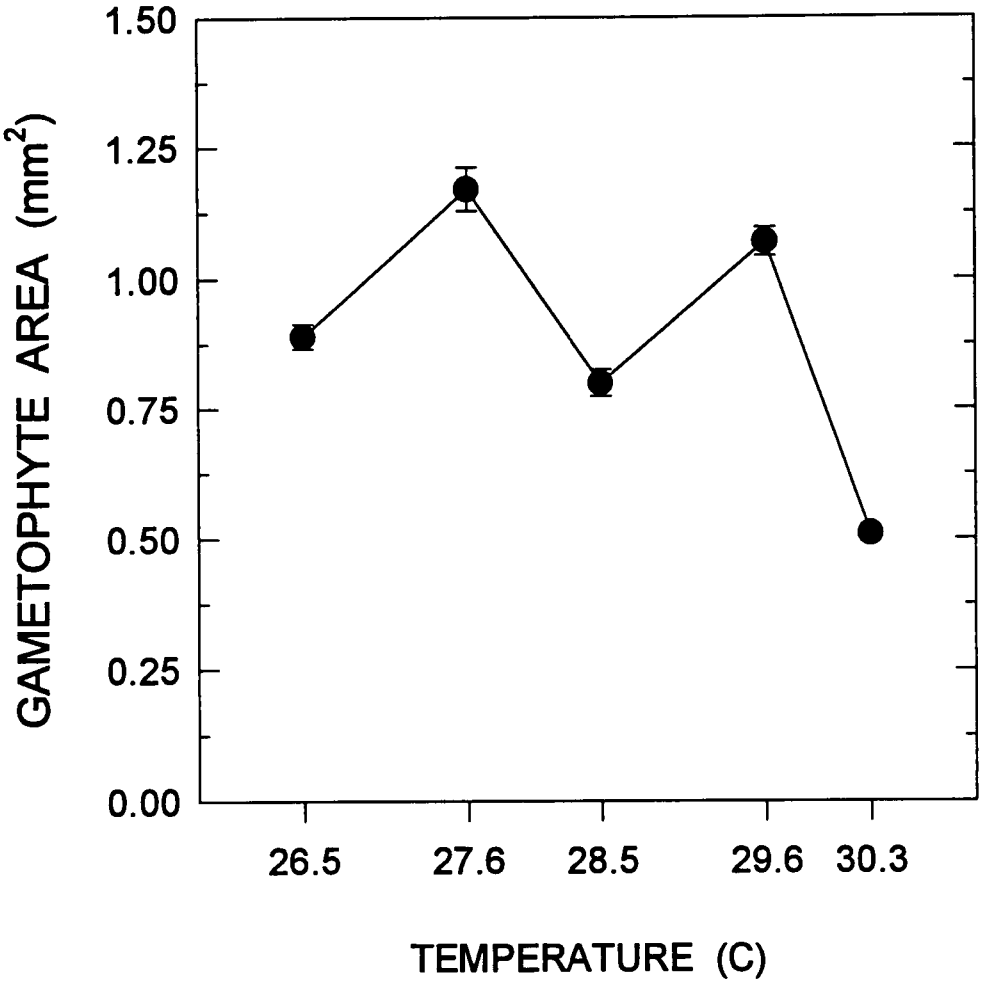

1.50
1.25
1.00
0.75
0.50
0.25
0.00
GAMETOPHYTE AREA (mm2)
26.5
27.6
28.5
29.6
30.3
TEMPERATURE (C)

Figure 21

Figure 22. Gametophyte area at 10 DFT of Hn cultured on various nutrient media solidified with washed Difco Bacto™ agar. Bars represent means ± standard error of 14-28 measurements. Tfhe horizontal line indicates the level of the Parker/Thompson nutrient media control.

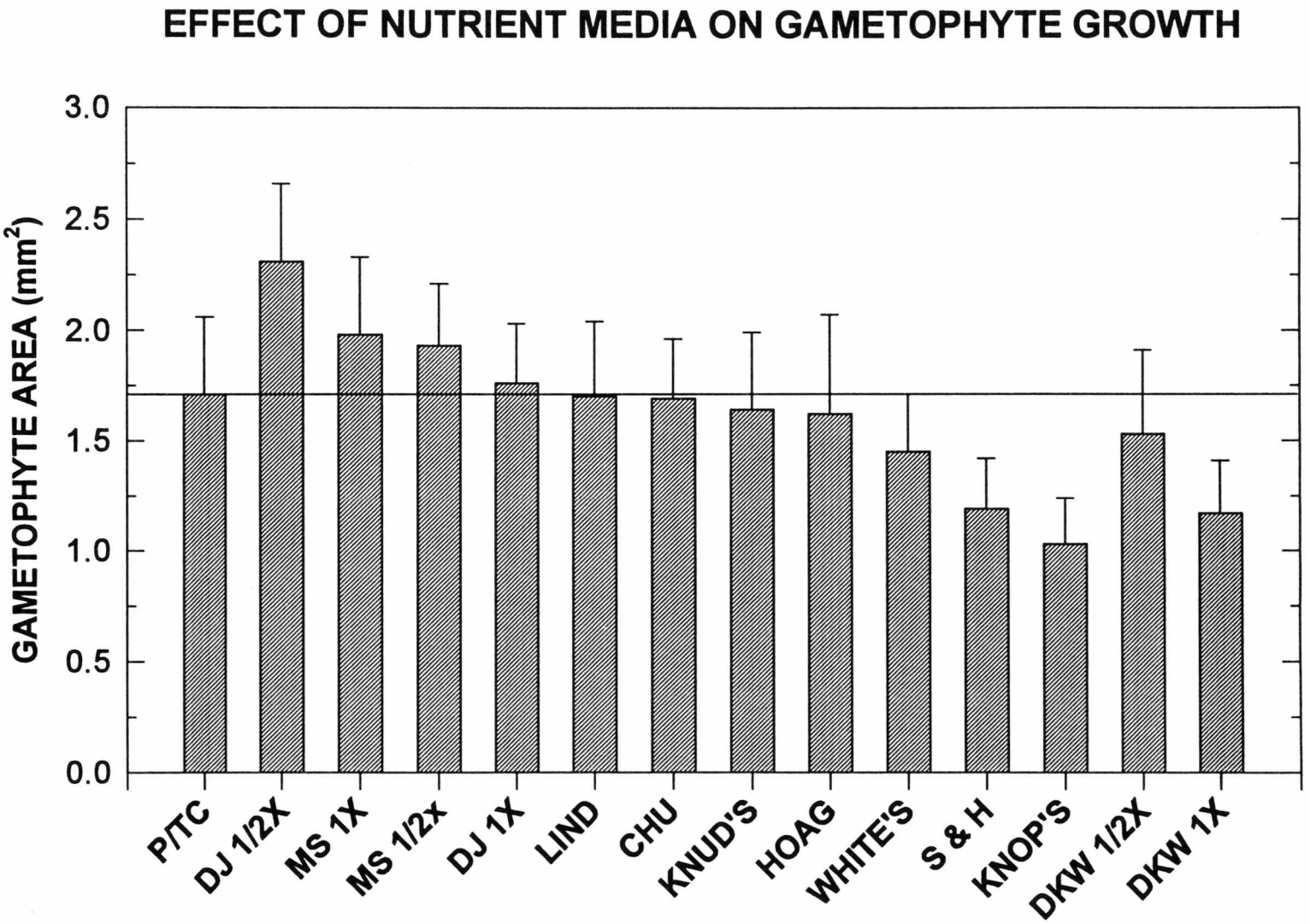

EFFECT OF NUTRIENT MEDIA ON GAMETOPHYTE GROWTH
GAMETOPHYTE AREA (mm²)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P/TC
DJ 1/2X
MS 1X
MS 1/2X
DJ 1X
LIND
CHU
KNUD'S
HOAG
WHITE'S
S & H
KNOP'S
DKW 1/2X
DKW 1X

Figure 22

Knop's, S&H, Chu and White's were all very pale in color (light grey). Because of the increased growth of gametophytes and the healthy long term growth of sporophytes on DJ 1/2X (data shown in Figure 14) this media was selected for use in the general culture of sporophytes.

VITA

Renée Sarah Albertine was born on October 11, 1962 in Knoxville, Tennessee. She received her equivalency diploma from Bearden High School in 1980. In 1987 she began studies at the University of Tennessee, Knoxville where she received her Bachelor of Science Degree in Ornamental Horticulture and Landscape Design in Summer 1992. The following semester she accepted a teaching assistantship at the University of Tennessee and began to pursue a Master of Science degree in botany. During her graduate studies she received the Raymond W. Holton Graduate Student Teaching Award. Ms. Fribourg will receive her Master of Science in Botany in August 1995.