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Relationship between turfgrass performance and low-temperature tolerance in perennial ryegrass /

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RELATIONSHIP BETWEEN TURFGRASS PERFORMANCE AND LOW-TEMPERATURE TOLERANCE IN PERENNIAL RYEGRASS

A Thesis Presented

by

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DEDICATION

To my wife, Pauline, whose love and support made this possible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Each winter, in New England and the northern United States, substantial turf losses occur due to injury resulting from freezing temperatures. In addition, 35 to 75% of all energy input associated with turf management is in the preparation for, and recovery from, winter (Rossi,1997). Nevertheless, extensive losses occurred as a result of the winters of 1992-93 in the midwest and 1993-94 in the northeast (Rossi,1997). Turf injury and losses that result from freezing temperatures can have an economic and environmental impact on the functional quality and aesthetic value of turf areas (DiPaola and Beard,1992). Turf loss results in increased weed pressure and herbicide cost, increased soil erosion, decreased use, and the need for extensive re-establishment procedures (DiPaola and Beard,1992; Rossi,1997).

Turfgrass species and varieties vary widely in their tolerance to freezing stress (Beard, 1973; Gusta et al., 1980). Perennial ryegrass (*Lolium perenne* L.) has been reported to have the poorest low temperature tolerance among cool-season turfgrass species (Beard, 1973; Gusta et al., 1980). Despite having a poor low temperature tolerance, perennial ryegrass is still one of the most widely used species in the northern United States (Meyer and Funk, 1989; Watson et al., 1992; Young and Baker, 1995). The ability of perennial ryegrass to establish quickly makes it a popular choice of turf managers for over-seeding fairways,

institutional grounds, parks, home lawns, and in lawn care operations (Beard, 1973). It is expected that the popularity of perennial ryegrass will increase in the northeast and elsewhere with the release of new and improved turf-type cultivars.

Turfgrass Freezing Stress

Turfgrass freezing stress occurs at 0° C and colder temperatures (DiPaola and Beard, 1992). As pointed out by Levitt (1980), the term freezing stress is technically a misnomer. In actuality, freezing is a strain resulting from a low-temperature stress. However, the term freezing stress has been accepted due to its general use in the literature (Levitt,1980).

Injury to turfgrass due to freezing temperatures involves the formation of ice crystals in and around the cells of the regenerative region of the plant (Beard, 1973; Levitt, 1980; Rossi, 1997). The regenerative region of a turfgrass plant, also known as the crown, is the region that includes the stem apex, the unelongated internodes, and the lower nodes from which the adventitious roots are initiated (Beard, 1973). Since the adventitious roots, lateral shoots (tillers, rhizomes, and stolons), and leaves all initiate from this region, the crown tissue is considered the most vital portion of a turfgrass plant (Beard, 1973). If temperatures drop quickly, intracellular freezing will occur in tissues having a high hydration level (Beard, 1973; Levitt, 1980; DiPaola and Beard, 1992; Rossi, 1997). The ice crystals cause a mechanical disruption to the protoplasm which usually results in death of the tissues (Beard, 1973; Levitt, 1980).

In nature, temperatures generally change slowly (1 to 2°C per hour) (Levitt, 1980; Rossi, 1997). Therefore, care must be taken with the use of simulation chambers in controlled environment studies to ensure that freeze-thaw conditions are realistic. The slow temperature change causes ice crystals to form first in the large vessels of the plant and then proceed to the intercellular spaces using the available water vapor and water film on cell walls (Levitt, 1980). This causes the vapor pressure in the intercellular space to drop below the vapor pressure within the cells (DiPaola and Beard; 1992). The differential in vapor pressure causes cell water to diffuse from the cells to the intercellular space, causing the ice crystal to grow, and the cells to dehydrate (Levitt, 1980; DiPaola and Beard, 1992; Palta and Weiss, 1993). It is this dehydration of the cells due to the extracellular freezing that causes freezing injury (Levitt, 1980; DiPaola and Beard, 1992).

The ability of turfgrass to survive is a function of the severity of injury and location of injury within the crown (DiPaola and Beard, 1992). If a sufficient number of cells within the crown of turfgrass plants are injured, the grass may not recover (Beard, 1973; Rossi, 1997). It has been reported that the lower portion of annual bluegrass crown is more likely to be injured, due to freezing stress, than the upper portion (Beard and Olien, 1963; Beard, 1973). Therefore, the lower apical meristem, responsible for root initiation, can be injured more easily than the upper apical meristem (Beard, 1973; Olien and Marchetti, 1976). This type of injury to turfgrass can be further exacerbated by desiccation in early spring when transpiration, resulting from warming temperatures and resumed

growth, can exceed the water uptake capability of the degenerating, injured, root system and lower crown tissue (Beard, 1973; DiPaola and Beard, 1992). The water absorption capability of a plant is predicated on the severity of injury to the lower portion of the crown and root system (Beard,1973). Tanino and McKersie (1985) stated that in winter wheat, survival to freezing stress was limited by the tolerance of a relatively small number of cells in the basal region (transition zone) of the crown rather than the apical meristem (shoot apices and lateral buds). Shibata and Shimada (1986) also showed greater susceptibility, to freezing-stress injury, of the transitional zone than the shoot apical meristem in orchardgrass (*Dactylis glomerata* L.). Whole-plant survival of bunch type turfgrass depends on the production of tillers and therefore, the improved survival of hardened perennial ryegrass depends on the ability to establish viable regrowth from lateral tiller buds (Eagles et al., 1993).

Turfgrass Freezing-Stress Resistance

Freezing-stress resistance as defined by Palta and Simon (1993) is the ability of the plant to survive freezing temperatures and maintain its functions which include its genetic potential for growth, development, and productivity. The ability of turfgrass to survive freezing stress is based on two survival mechanisms, avoidance and tolerance (Levitt, 1980; DiPaola and Beard, 1992).

Avoidance

Avoidance to freezing stress results when either the exposure is prevented or when it is reduced (DiPaola and Beard, 1992). In cold climates,

warm-season turfgrass growing near heat sources like buildings, heat vents, and underground pipes used for heating or ventilation is an example of freezing-stress exposure prevention.

Supercooling is an important freezing-stress avoidance mechanism used by many woody plants (Levitt, 1980). However, DiPaola and Beard (1992) have stated that freezing-stress avoidance by supercooling has no known significance to turfgrass. The depression of the freezing point in cells, due to the accumulation of carbohydrates and other solutes during the cold acclimation period, reduces a plant's exposure to freezing stress (Beard, 1973; Levitt, 1980). However, other researchers have observed only a slight (to -4° C) depression in the freezing point and, therefore, by itself, the concentration of solutes is not viewed as an important freezing-stress-avoidance mechanism in turfgrass (Levitt, 1980; Williams, 1980; Thomas and James, 1993; Rossi, 1997).

Tolerance

The ability of a plant to survive a freezing stress can be defined as its tolerance to the stress. Hardiness is a commonly used term to imply tolerance or acclimation (DiPaola and Beard, 1992). It is the ability of the plant to survive an unfavorable internal environment (Levitt, 1956). Turfgrasses used in cold climates must possess cold hardiness in order to survive periods of low - temperature stress (DiPaola and Beard, 1992). Since intracellular freezing in turfgrass almost always results in death to the plant, freezing-stress tolerance then implies tolerance to extracellular ice formation and the resulting dehydration (Levitt, 1980).

Cold Acclimation

A plant's capacity to cold acclimate and then deacclimate has long been considered to be significant in determining freezing tolerance (Carroll, 1943; Gay and Eagles, 1991; Fry et al., 1993; Rossi, 1997). The degree of cold hardiness varies throughout the winter (Beard, 1966). Although peak hardiness is generally achieved in early winter, it typically begins to decrease in February and is drastically reduced by late winter (Beard, 1973). Investigation into cold tolerance of four species of turfgrass including Kentucky Bluegrass (*Poa pratensis* L.), perennial ryegrass, creeping red fescue (*Festuca rubra* L.), and creeping bentgrass (*Agrostis palustris* Huds.) in Minnesota (White and Smithberg, 1980), indicated that the acquisition of cold tolerance started as early as July and peaked in January. By February, the loss of acclimation had begun, and as ambient temperatures approached the freezing mark, the ability to tolerate freezing stress was rapidly being lost (White and Smithberg, 1980). Cold acclimation was lost at a faster rate in the spring. It is during the spring transitional period that the majority of low-temperature injury to turf occurs (Beard, 1973; Rossi, 1997). At this time, wide fluctuations in temperature can stimulate growth and cause an increase in the hydration level of crown tissue, then, if temperatures drop below the tolerance level of the plant, serious injury can result (Beard, 1973; White and Smithberg, 1980; Rossi, 1997).

Factors Influencing Low-Temperature Hardiness

Cold acclimation (hardiness) in turfgrass involves physiological changes within the plant. As fall soil temperatures approach 7°C, turfgrass shoot growth

slows and eventually stops (Beard, 1973). Carbohydrate levels increase and protoplasm hydration levels decrease, resulting in the tissue achieving a maximum level of low-temperature hardiness (Beard, 1973; Levitt, 1980; Rossi, 1997). Low-temperature hardiness is inversely correlated with the seasonal changes in crown tissue hydration levels (Beard, 1973; Levitt, 1980). A period of 3-4 weeks of average daily air and soil temperatures between 1°C to 5°C are optimum to harden cool-season turfgrasses (Beard,1973).

Cultural Influences On Winter Hardiness

The plant growth process uses up stored carbohydrates and increases the hydration level of cells, therefore, actively-growing plants have a minimum low-temperature tolerance (Beard, 1973). Accordingly, cultural practices that stimulate growth reduce the low-temperature hardiness of turfgrass (Beard, 1973). Some cultural practices that decrease the cold-temperature-tolerance level include (i) failure to provide proper drainage, (ii) nitrogen applied in excess or in late fall, (iii) inadequate applications of potassium, (iv) excessive late fall irrigation, (v) lack of thatch control, and (vi) a close cutting height (Beard, 1973).

Relationship Between Freezing-Stress Tolerance and Turfgrass Quality Performance

Freezing-stress tolerance is a major factor limiting the adaptation of turfgrass to northern climates (Beard, 1973). For species with marginal low - temperature hardiness, such as perennial ryegrass, this may be an especially important limitation. Studies to determine both the extent of freezing-stress tolerance as a limiting factor to the adaptation of perennial ryegrass in northern

New England and the northeast, and its contribution to turfgrass quality performance, is limited. Turfgrass quality is a subjective measurement of aesthetic appeal and functional value and includes characteristics such as color, shoot density, uniformity, and texture (Turgeon,1980). Turfgrass shoot density is the single most important component of turfgrass quality (NE 57 Technical Research Committee, 1977). Hence, a significant correlation between turfgrass quality and shoot density has been reported (NE 57 Technical Research Committee, 1977). In grass, whole-plant survival to freeze-stress temperatures depends on tiller production for regrowth (Eagles et al., 1993). The vegetative shoot (tiller) consists of a short central stem of unelongated internodes with leaves borne alternately at successive nodes (Beard, 1973). Eagles et al. (1993) have suggested that superior tillering could have a beneficial effect on the recovery from low-temperature stress, because more tiller buds are available for regrowth, thus influencing winter survival. Superior turfgrass performers could have improved freeze-stress recovery and survival because of more profuse tillering compared to poor turfgrass performers. Accordingly, there is a potential link between turfgrass quality and the capacity to recover from freeze-stress injury. It is important to recognize, however, that low-temperature kill (freeze stress) is only one of several potential mechanism of injury operating that may limit turfgrass survival. Other mechanism of injury of cool-season turfgrass include low-temperature disease, ice encasement (suffocation), desiccation, and traffic (Beard, 1973; Blum, 1988; Humphreys, 1989).

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CHAPTER 2

RELATIONSHIP BETWEEN TURFGRASS PERFORMANCE AND LOW-TEMPERATURE TOLERANCE IN PERENNIAL RYEGRASS

Abstract

Turf losses from freeze-stress injury result in costly re-establishment especially with turfgrass species such as perennial ryegrass (*Lolium perenne* L.) having poor low-temperature tolerance. Research studying the relative importance of low-temperature tolerance and its contribution to turfgrass quality performance in northern climates is limited. The objectives of this research were to compare critical freezing temperature thresholds of ten perennial ryegrass cultivars representing contrasting turfgrass quality types (5-high and 5-low performance cultivars). The criteria for cultivar selection was based on the relative turfgrass quality rank (top and bottom five) from a National Turfgrass Evaluation Program (NTEP) trial conducted at the Maine (Orono) location (the most northern NTEP location in New England). Ten freeze-stress temperatures (-3, -5, -7, -9, -11, -13, -15,-17, -19, and -21°C) and a non-frozen control (+5°C) were evaluated including acclimated (AC) plant material that was acclimated outside in an unheated polyhouse during the fall and winter in Massachusetts and non-acclimated (NA) plant material (greenhouse grown). Low-temperature tolerance was assessed using whole-plant, freeze-shock survival and electrolyte leakage (EL) methods. Lethal killing temperatures LT_{50}, derived from fitted EL

and survival curves were determined. The high-performance (HIGH) cultivars were able to tolerate significantly lower freeze-stress temperatures, indicated by less EL and superior whole-plant survival, compared to low-performance (LOW) cultivars. Based on survival, $LT_{50\ HIGH}$ for AC tissues ranged from -9.3 (Prelude III) to -14.7°C (LRF-94-C8), and $LT_{50\ LOW}$ ranged from -3.0 (Linn, SR-4010) to -11.8°C (Pennfine). Based on EL, $LT_{50\ HIGH}$ for AC tissues ranged from -3.4 (Top Hat) to -6.7°C (LRF-94-C8), and $LT_{50\ LOW}$ ranged from -0.5 (SR-4010) to -5.8°C (Pennfine). The lowest cultivar LT_{50} for NA tissues was only -4.2°C (Prelude III) and was based on survival. The EL method was shown to have good predictive value ($r=0.80$, $p \leq 0.01$) for whole-plant survival in AC tissues. Slope estimates from EL curves indicated that significantly lower slopes (lower mortality rates) were detected with AC tissue (compared with NA) and with HIGH cultivars (compared with LOW cultivars). These results underscore the importance of selecting low-temperature tolerant perennial ryegrass because superior low-temperature survival is associated with superior turfgrass performance in adapting to northern New England.

Introduction

Turf losses due to freezing injury result in costly re-establishment, increased weed pressure and related herbicide cost, increased soil erosion, and a general reduction in the aesthetic value and function of turf areas (DiPaola and Beard,1992). Turfgrass species and cultivars within species vary widely in their tolerance to freezing stress (Beard, 1973; Gusta et al., 1980). The cold

tolerance among nine cool season turfgrass species to lethal temperatures, as reported by Gusta et al. (1980), ranged from -35°C for creeping bentgrass (*Agrostis palustris* Huds.) to -5 to -15°C for cultivars of perennial ryegrass.

Freezing-stress tolerance is an important factor limiting the adaptation of turfgrass to northern regions of the United States (Beard, 1973). This may be an especially important limitation for those species, such as perennial ryegrass, having only marginal low-temperature hardiness. Despite perennial ryegrass having the poorest low-temperature tolerance among cool-season turfgrass species, it is still one of the most widely used species in the northern United States (Meyer and Funk, 1989; Watson et al., 1992; Young and Baker, 1995). The popularity of perennial ryegrass for over-seeding fairways, institutional grounds, parks, home lawns, and in lawn care operations is due largely to its ability to establish quickly (Beard, 1973). It is expected that this popularity will continue to increase with the release of improved turf-type cultivars.

Studies to determine the extent of freezing-stress tolerance as a limiting factor to the adaptation of perennial ryegrass in northern New England and its contribution to turfgrass quality performance are limited. In a review of temperature stress (DiPaola and Beard, 1992), 85% of the cited research was based on work done with cereal grains and forage grasses, plants related to, but perhaps not comparable to, turfgrass species (Rossi,1997). There are no published reports relating low-temperature hardiness to turfgrass performance.

In grass, whole-plant survival to freeze-stress temperatures depend on tiller production for regrowth (Eagles et al., 1993) from lateral tiller buds. It has

been suggested by Eagles et al. (1993), that superior tillering could have a beneficial effect on plant recovery from low temperature because more tiller buds are available for regrowth, thus influencing winter survival. Superior turfgrass performers could have improved freeze-stress recovery and survival because of greater capacity for tillering compared to poor turfgrass performers. Accordingly, there is a potential link between turfgrass quality and the capacity to recover from freeze-stress injury. It is important to recognize, however, that low-temperature kill (freeze stress) is only one of several potential winter stresses operating that may limit turfgrass survival. Winter kill is the result of a combination of factors including low-temperature desiccation, low-temperature diseases, ice encasement (suffocation), and cultural factors (Beard, 1973; Blum, 1988; Humphreys, 1989).

Evaluation of recovery (survival) after low-temperature exposure is a reliable method for assessing low-temperature tolerance (Steponkus, 1978). However, this procedure is time consuming when dealing with plants that require a lengthy period for re-establishment before they can be assessed, and normally does not provide information regarding the mechanisms that cause death of the freeze-stressed plant (Cardona et al., 1997). To assess the level of cell injury due to low-temperature stress, the electrolyte leakage technique is commonly used (Palta, 1980; Cardona et al., 1997). The technique has been used extensively with woody species (Furmanski and Buescher, 1979; Lu and Rieger, 1990), succulent plants (King and Ludford, 1983; Sulc et al., 1991; Manley and Hummel, 1996), and cool and warm season grasses (Gusta et al., 1980;

Rajashekar et al.,1983; Cohen and Wood, 1986; Fry et al., 1993). The electrolyte leakage method has been shown to have good predictive value for lethal low temperatures based on whole-plant, freeze-shock survival and has been suggested as a method to screen for freeze-stress tolerance in turfgrass (Gusta et al., 1980; Murdoch et al., 1990; Fry et al., 1991; Maier et al., 1994; Cardona et al., 1997). However, a high leakage level may not always equate to higher membrane injury when plants are allowed to acclimate (Uemura and Steponkus,1994; Uemure et al. 1995). Therefore, survival evaluations should be included with electrolyte leakage to provide a more reliable assessment of low-temperature survival.

The objectives of this research were to compare critical freezing temperature thresholds of ten cultivars of perennial ryegrass representing contrasting turfgrass quality types, under acclimated and non-acclimated conditions, using whole-plant survival and EL methods.

Materials and Methods

Plant Material Selection

The criteria for cultivar selection was based on the relative ranking from the most recent (1997) National Turfgrass Evaluation Program (NTEP) field trial conducted at the Maine (Orono) location (the most northern NTEP location in New England) (USDA; 1997). Five cultivars: LRF-94-C8, Palmer III, Prelude III, Repell III, and Top Hat, representing the high performers, and five cultivars: DSV NA 9401, DSV NA 9402, Linn, Pennfine, and SR-4010 representing the low

performers were selected. Accordingly, genotypes representing "HIGH" and "LOW" performance types were one of the treatment factors evaluated.

The average January air temperature for Maine during the period from 1994 to 1997 (the NTEP evaluation period on which the selection was based) was -10.3°C (NRCC report). Air temperature is less important to crown survival than soil temperature since crowns are located near or below the soil surface and are protected by the warmer soil temperatures (Beard, 1973). The temperature data indicates, however, that the average temperature for January in Maine is within the critical lethal temperature for some perennial ryegrass cultivars.

Pure (authentic) seed from each cultivar was obtained directly from the breeder. Plants were established from seed (seeding rate, 439 kg ha^{-1}), under mist in the greenhouse, beginning 23 Sep. 1998 through 2 Oct. 1998. Seed was sown in 5 cm diameter by 17.8 cm deep pots filled with a commercial planting mix consisting of peat, perlite, and vermiculite (Sierra Customblen Bale Mix, Scotts, Marysville, OH). A control release fertilizer (Osmocote 14-6.2-11 [N-P-K], Scotts, Marysville, OH) was incorporated at a rate of 146 kg N ha^{-1} into the planting mix at planting time. Soil pH of the potting mix was 5.9 out of the bag. Nutrient levels (macro and minor elements) were normal based on soil test results.

Acclimation Conditions

Once plants reached the 4^{th} leaf stage (between 13 Oct. 1998 and 19 Oct. 1998), half of the containers of each cultivar were moved to a cool greenhouse

(14 to 17°C average daily temperature). This environment allowed the plants to grow and develop and to begin to acclimate to a cooler temperature before being placed in the field. The second half of the containers were moved to a warm greenhouse (18 to 22° C average daily temperature) representing "Non-Acclimated" conditions. Genotypes representing "LOW" and "HIGH" performance levels were also evaluated under two environments representing "Acclimated" and "Non-Acclimated" tissue. All plants were watered as needed and "mowed" weekly to approximately 5 cm above soil level. The greenhouse temperatures were monitored hourly using an air/gas thermocouple attached to a printing thermometer (model 422314, Extech Instruments, Cole Palmer, Vernon Hills, IL).

On 9 Dec. 1998, container plants from the cool greenhouse were transferred to the field and placed in a cold frame in a covered but open-ended polyhouse at the University of Massachusetts Turfgrass Research Farm in South Deerfield, Massachusetts. Containers were placed directly on the soil surface, configured as a block by cultivar, and placed as close to each other as possible. Corning, 3½", home building thermal insulation (R13) was wrapped around the periphery of the containers in order to maintain the root-zone temperatures as close to natural in-ground temperatures as possible. The plants were kept here, and maintained for the remainder of the fall season into winter, in order to simulate field-acclimation conditions as closely as possible. Air, crown, and root-zone temperatures were monitored using copper-constantan thermocouples connected to a datalogger (21X, Campbell Scientific, Inc., Logan, UT).

Thermocouples were also placed outside the polyhouse on the soil surface and 2.5 cm into the soil. Figure 2.1 shows the mean daily air temperature for both acclimated and non-acclimated environments for the duration of the experiment and Fig. 2.2 shows the mean daily soil temperature vs. the plant container media temperature at the surface and 2.5 cm into the media.

Light conditions were measured on a bright, cloudless day at the beginning of the experiment (1 Oct. 1998) and at the end of the experiment (17 Mar. 1999) using an Integrating Quantum/Radiometer Photometer (LI-188B, LI-COR Inc. Lincoln NE). The mean value of measurements made across the plants in the greenhouse (NA material) in October was 2119 ±347 (mean ±SD, n = 8) μmol m^{-2} s^{-1} Photosynthetically Active Radiation (PAR) and in March was 2737 ±633 (mean ±SD, n = 8) μmol m^{-2} s^{-1} S.D. PAR. A measurement made outside the greenhouse was 3840 μmol m^{-2} s^{-1} PAR in October and 3570 μmol m^{-2} s^{-1} PAR in March.

Freeze-Shock Recovery (Survival) Evaluation

Freezing shock and subsequent recovery of plant material was evaluated by first submitting samples to a range of 11 decreasing treatment temperatures consisting of a non-frozen control (+5°C), and 10 freeze-stress temperatures: -3, -5, -7, -9, -11, -13, -15,-17, -19, and -21°C. Treatment temperatures were applied using a ScienTemp$_{TM}$ freezer (Scientemp Corp. Adrian, MI).

Eleven randomly-selected replicates (container plants) from each of the 10 genotypes of acclimated and non-acclimated plants (total of 220 containers)

were evaluated together. Samples from one replicate from each cultivar-environment combination (total of 20 containers) were prepared for each treatment temperature. The temperature of the plant material was cooled to the desired treatment level at a rate of 2°C h^{-1} maximum. The material was then allowed to remain (soak) at that temperature for a minimum of 1h. After temperature exposure the plant material being evaluated were removed from the freezer and assessed for survival as a percentage of viable-green shoots. The temperature at which 50% of the crown tissue survive based on regrowth recovery was determined, and expressed as LT_{50}. This procedure was repeated for a total of four replications.

There were 880 experimental units corresponding to 10 (cultivars) x 2 (environments) x 11 (temperatures) x 4 (replications) used for the freeze-stress survival evaluation. Acclimated plant samples were taken from plant material maintained outside, at the South Deerfield farm, during early February and March.

Plant material was washed free of soil, using cold water, and separated into individual plants. Ten individual plant samples from each replicate plant container were prepared for treatment at each temperature. Shoots and roots were trimmed to 2 cm each. To ensure ice nucleation, samples were wrapped in paper toweling moistened with deionized water and placed into poly freezer bags for freeze stress treatment. During sample preparation all plant material was temporarily stored at 5°C, non-frozen control temperature.

Freezing schedule and Freeze-Shock Recovery. After preparation was completed, all the freezer bags containing the samples were placed in the freezer at the same time and left overnight at -2°C in preparation for the freeze-stress temperature schedule. Non-frozen control samples remained overnight at +5°C. Treatment temperatures were adjusted manually and monitored using six copper-constantan thermocouples, attached to a datalogger (CR10X, Campbell Scientific, Inc., Logan, UT). The thermocouples were placed in freezer bags containing left over plant material and then selectively placed in the freezer adjacent to plant tissue under evaluation. Treatment temperatures -3 to -11°C were applied the first day. The freezer was then left at -11°C overnight and the remainder of the freezing schedule was completed the following day. After each treatment-temperature exposure, freezer bags containing samples from each cultivar-environment combination (total of 10 acclimated and 10 non-acclimated bags per treatment temperature) were removed from the freezer and allowed to thaw slowly, for a minimum of 12 h, at +5°C. Samples were then replanted in cell trays, and placed in the greenhouse for a four week recovery period (i.e. survival evaluation). Plants with any green surviving tissues or any new growth from even one shoot were counted as survivors. All others were considered as having been killed by the treatment temperature. Percent survival was calculated as: Survival (%) = (no. of plants survived ÷ total no. of plants) x 100.

Electrolyte Leakage Evaluation

Electrolyte leakage evaluation was conducted to assess the level of crown tissue injury caused by freezing stress. Methods similar to those

described by Cardona et al. (1997) were used. Acclimated and non-acclimated container plants from each of the ten genotypes were evaluated together at each of the temperature levels as described above in the freeze-shock recovery evaluation. After temperature exposure the samples were removed from the freezer and an initial electrical conductivity (EC) of crown tissue was measured. The tissue was then killed at -40°C and a final EC measurement made and compared with the initial EC for the same tissue. The ratio of the two readings, expressed as a percentage, serves as an index of electrolyte leakage and cell integrity in response to temperature stress. The procedure was repeated for a total of three replications. The temperature at which 50% leakage occurred was expressed as LT_{50}. This was compared with the LT_{50} determined from the regrowth evaluation.

There were 660 experimental units corresponding to 10 (cultivars) x 11 (temperatures) x 2 (environments) x 3 (replications) used for the electrolyte leakage assay. Acclimated plant samples were taken from plant material maintained outside, at the South Deerfield farm, during early February and March.

Plant material was washed free of soil, using cold water, and separated into individual plants. Fifteen individual plant samples from each replicate plant container were prepared for treatment at each temperature. Leaves and roots were removed leaving only crowns. Five crown samples were placed into each of three test tubes and stoppered. To ensure ice nucleation, test tubes were pre filled with 4 ml deionized water, frozen, and held at -2°C before plant material

was added. Crowns were then placed in the test tubes in contact with the ice and an additional 1 ml deionized water was added. During sample preparation all plant material was temporarily stored at +5°C, non-frozen control temperature.

Freezing schedule and Electrolyte Leakage Assay. After preparation was completed, all the test tubes containing the crown samples were placed in the freezer at the same time, and left overnight at -2°C, in preparation for the freeze-stress temperature schedule. Non-frozen control samples remained overnight at +5°C. Treatment temperatures were adjusted manually and monitored using six copper-constantan thermocouples, attached to a datalogger (CR10X, Campbell Scientific, Inc., Logan, UT). Thermocouples were placed in test tubes containing left over crown tissue, and selectively placed in the chamber adjacent to crown tissue under evaluation. Treatment temperatures -3 to -11°C were applied the first day. The freezer was then left at -11°C overnight and the remainder of the freezing schedule was completed the following day. After each treatment-temperature exposure three test tubes, each containing five crown tissues from each cultivar-environment combination (total of 30 acclimated and 30 non-acclimated test tubes per treatment temperature), were removed from the freezer and allowed to thaw slowly, for a minimum of 12 h, at +5°C. Samples were then subjected to infiltration (test tubes uncovered) under partial vacuum for 20 min, then incubation (test tubes covered) at +5°C for 12h, and then placed on a shaker table (approximately 150 rpm) at room temperature for 8h. Initial conductivity (IC) of the extract of each test tube was measured, using a conductivity bridge (Model 1054, VWR Scientific, Boston, MA). Crowns were

then killed by way of overnight exposure to -40°C. Samples were thawed at room temperature for 4h minimum and placed on the shaker table for 2h at room temperature after which the final conductivity (FC) of the extract was measured. Electrolyte leakage (EL) is expressed as a relative percentage:

$$EL\ (\%) = (IC \div FC) \times 100$$

Scheduling Freeze-Shock Recovery and Electrolyte Leakage Evaluations

It takes five days plus a day for preparation to complete a freeze-shock recovery assessment and seven days plus a preparation day to complete an electrolyte leakage assay. Due to the shear number of samples, the number of replications, and the physical size of the freezer and support equipment, it was necessary to partition the testing work load by method and replication. The total time from start to finish for the assessment of all replications of freeze-shock recovery and electrolyte leakage was seven weeks. It would have been preferred to evaluate both freeze-shock recovery and electrolyte leakage simultaneously rather than separately. Time delay between assessment methods could introduce potential differences in acclimation (or de-acclimation), that may occur in the plant material being maintained outside as the source of acclimated material. In order to minimize this effect, the replications (blocks) of the individual assessments were alternated between survival and EL evaluations. Consequently, time was used as a blocking variable.

Statistical Analysis

Analysis of variance (ANOVA) was performed by MINITAB (State College, PA) and non-linear regression using SPSS Advanced Statistic (SPSS Inc., Chicago, IL). Cultivar sum of squares were partitioned into single degree of freedom (df) contrast to test for the difference between the combined means comparing HIGH vs. LOW turfgrass performance types (Tables 2.1 and 2.2). Additionally, contrast among HIGH and LOW cultivar types were compared to test for difference within turfgrass performance cultivars. Ten cultivars, two environments, and eleven temperature levels were analyzed as fixed effect treatment factors using a complete factorial arrangement in a randomized complete block design. No serious departures from the assumptions of ANOVA were detected in homogeneity of variance or normality of errors.

A curve was fitted using non-linear regression to mean EL by cultivar (Fig. 2.3 through 2.12) and turfgrass performance type (Fig. 2.13 and 2.14) using a similar method as described elsewhere (Ingram, 1985; Anderson et al., 1988; Cardona et al., 1997). The general equation was:

$$El_p = El_l + [(El_m - El_l)/(1 + e^{-B(T-Tm)})] \quad \text{Eq. [1]}$$

where El_p = predicted EL; El_l = lower bound EL value; El_m = upper bound EL value; e = 2.714; *B* is the rate of increase of the slope of the curve; *T* is the absolute value of the treatment temperature, and T_m = inflection point of the curve and is defined as the midpoint between the lower and upper asymptote of

the curve (Zhu and Liu, 1987). The inflection point of the curve is closely correlated to the lethal killing temperature of the tissue. However, T_m frequently overestimates actual low temperature tolerance (von Firks and Verwijst, 1993; Fry et al.,1993; Maier et al., 1994; Cardona et al., 1997) and will not be reported here. The parameter of special interest was the slope of the curve (*B*), which has been described as an important indicator of mortality resulting from low-temperature stress (Gudleifsson et al., 1986; Zhu and Liu, 1987; Cardona et al.,1997). Absolute values of temperature were used in curve fitting to estimate *B*. To avoid introducing two values for 5°C (+5°C non-frozen control and the -5°C treatment temperature) the non-frozen control was omitted, consequently -3°C was the highest temperature evaluated using Eq. 1. Given that *T* ranged from -3 to -21°C, only a portion of the EL curve corresponding to the slope could be estimated, consequently the slope estimates are approximations.

Lethal temperatures at which 50% survival and EL occurred (i.e., LT_{50}) was determined mathematically by curve fitting using a four parameter sigmoid model (Sigma Plot, SPSS Inc., Chicago IL). Specifically, parameter estimates were substituted back into the non-linear equation and the temperature where Y (survival or EL) is 50% was determined for each cultivar (Fig. 2.3 through 2.12 and Tables 2.3 and 2.4) and turfgrass performance type (HIGH vs. LOW) (Fig. 2.13 and 2.14) for acclimated and non-acclimated tissues. Curves shown in all figures were fitted accordingly to mean EL or survival with eleven treatment temperatures. Equivalently, a dashed line where Y equals the 50% response level is included with all EL and survival curves, with vertical lines intersecting

the 50% level to estimate the lethal temperature (LT_{50}) for each cultivar and performance group. The survival and EL data shown in all figures (2.3 through 2.14) followed a sigmoidal response, typical for this type of experiment (Zhu and Liu, 1987; Anderson et al., 1988; von Fircks and Verwijst, 1993; Anisko and Lindstrom, 1995; Cardona et al., 1997). Simple correlation coefficients (Snedecor and Cochran, 1989) were computed for cultivar LT_{50} (Table 2.5) to compare the relative agreement and predictive value between acclimated and non-acclimated tissue types.

Results and Discussion

Based on accumulated clippings, it was observed that plants placed in the cool greenhouse grew at a slower rate than the plants growing in the warm greenhouse. This may have signaled the beginning of acclimation due to cooler day-night temperatures. Once the plants from the cool greenhouse were moved to the cold frame in the field, measurable growth ceased. Over the following weeks a change in leaf color of the AC plants was observed, first to yellow then by the end of the testing period brown, indicating that dormancy had set in or injury had occurred. For several days between the time when the plants were first set out (on 9 Dec. 1998) and 1 Jan. 1999, the average daily air temperature, measured 30 cm above the plants, was below -3°C (Fig. 2.1). The potentially short acclimation period (only 14 days before temperatures fell below freezing) used in this experiment tested the ability of perennial ryegrass cultivars

to acclimate quickly. The average air temperature for the first three days in January, just three weeks after plants were set out, was between -9.0°C and -12.1°C and on 15 January the average temperature for the 24-h period was -14.8°C. The average daily media temperature, just at the surface, was between -5.2°C and -7.4°C for the first three days in January respectfully and -12.6°C on 15 January (Fig. 2.2). Such cold temperatures, before the plants had sufficient time to fully harden (Beard, 1973), was cause for concern.

As noted previously, time was used as a blocking variable. Blocking was shown to have a significant effect within each method of assessment (Tables 2.1 and 2.2) so blocking was effective in accounting for variation due to time. Cultivars (HIGH vs. LOW), environment (AC and NA), and temperature were important main effects affecting low-temperature hardiness based on survival (Table 2.1) and EL (Table 2.2) evaluations. Differences between cultivars in whole-plant freeze-shock recovery and leakage were dependent on environment (AC and NA), as indicated by a significant cultivar (C) by environment (E) interaction (Tables 2.1 and 2.2.) Accordingly, the relative ranking of cultivars in freeze-stress tolerance (survival and EL) varies with environment and was dependent on whether plants had been allowed to adjust to low temperature through acclimation.

The acclimated cultivars exhibited greater freezing-stress tolerance than the non-acclimated cultivars, as indicated by significantly lower LT_{50} estimates (Tables 2.3 and 2.4). For survival data, AC tissues had a mean LT_{50} of -8.4°C compared to NA plants which had a mean LT_{50} of only -1.8°C (Table 2.3). The

range in cultivar LT_{50} was greater for AC plants (-3.0 to -14.7°C) compared to NA (+3.9 to -4.2°C). Gusta et al. (1980) reported a similar range (-5 to -15°C) in LT_{50} for acclimated perennial ryegrass cultivars based on regrowth-survival studies. The outcome of regrowth was visually dramatic. After four weeks of regrowth for each replication, a visual count of the acclimated plants showed that some plants from a few cultivars had survived after exposure to temperatures as low as -21°C. However, there were no survivors of the non-acclimated plants exposed to temperatures lower than -7°C. Based on EL evaluations, AC plants also tolerated significantly lower freezing temperatures compared to NA tissue (Table 2.4). For example, AC plants had a mean LT_{50} of -4.4°C compared to mean estimates of -0.6°C for NA tissue. The range in cultivar LT_{50} estimates based on EL evaluations were smaller for AC (-0.5°C to -6.7°C) and NA (1.6°C to -2.6°C) tissues compared to those observed for freeze-shock survival. The LT_{50} estimates based on survival were lower (more negative) compared to those implied based on LT_{50} estimates derived from fitted EL curves. Hence, EL may underestimate actual survival. In paspalum (*Paspalum vaginatum* Swartz), Cardona et al. (1997) also observed lower LT_{50} estimates based on regrowth survival compared to membrane leakage assays. While low-temperature tolerance suggested from LT_{50} estimates for perennial ryegrass survival and EL methods differ substantially in their absolute values, there was some agreement in cultivar LT_{50} estimates between survival and EL results. Specifically, for AC plants, the EL_{50} estimates derived from EL curves were correlated with those determined based on survival ($r = 0.80$, $p \leq 0.01$; Table 2.5). Consequently, EL

derived LT_{50} estimates of AC material have good predictive value for cultivar low-temperature survival. Accordingly, EL has been suggested as a potential screening method for improved low-temperature tolerance (Gusta et al., 1980; Murdoch et al., 1990; Fry et al., 1991; Maier et al., 1994; Cardona et al., 1997).

As a group, the high-performance cultivars were able to tolerate significantly lower freeze-stress temperatures than low-performance cultivars. Based on LT_{50} estimates the high-performance cultivars had less leakage and superior whole-plant survival, compared to the low-performance cultivars as indicated by significantly lower LT_{50} temperatures (Table 2.3 and 2.4). For AC plants, the HIGH performance cultivars had a mean LT_{50} of -10.9°C compared to LOW cultivars which had a mean of -6.0°C based on survival (Table 2.3). Also, visual differences between cultivar groups were stricking after four weeks of regrowth. No difference between performance groups was detected in survival for NA plants. These results suggest that differences in survival between contrasting turf quality types may only be detected when cultivars are allowed to adjust to low temperature through acclimation.

Based on survival, $LT_{50\ HIGH}$ for AC tissues ranged from -9.3 (Prelude III) to -14.7°C (LRF-94-C8), and $LT_{50\ LOW}$ ranged from -3.0 (Linn, SR-4010) to -11.8°C (Pennfine). The cultivars Linn and SR-4010 experienced high mortality rates (low survival) at +5°C (the non-frozen control) following acclimation in the cold frame. Specifically, low survival rates corresponding to 34 and 42% were observed for Linn and SR-4010 (Fig. 2.10 and 2.12, respectively). As a result, LT_{50} values could not be calculated, so these cultivars were arbitrarily given LT_{50}

temperatures corresponding to the highest freeze stress treatment (-3.0°C). The low temperatures recorded in the cold frame during January (Fig. 2.1 and 2.2) probably were approaching critical lethal temperatures for these two cultivars. Linn perennial ryegrass has been reported to be susceptible to low-temperature injury based on its high LT_{50} temperature of -6.0°C (Gusta et al.,1980) following acclimation. The soil media temperatures in January, in this study, (Fig. 2.2) of -12.6°C probably exceeded Linn's critical threshold and consequently high mortality rates were observed during regrowth studies. Interestingly, Linn and SR-4010 perennial ryegrass are members of the LOW performance group and were the only cultivars which suffered mortality rates greater than 50%, even at the non-frozen control temperature. These results underscore the superior low-temperature survival associated with HIGH performance cultivars, indicated by the significant ($p \leq 0.001$) single df contrast (comparing HIGH and LOW cultivars) for whole-plant survival (Table 2.1).

Based on EL, $LT_{50\ HIGH}$ for AC tissues ranged from -3.4 (Top Hat) to -6.7°C (LRF-94-C8) and $LT_{50\ LOW}$ ranged from -0.5 (SR-4010) to -5.8°C (Pennfine). The low-performance cultivar Pennfine had LT_{50} values (i.e. low-temperature tolerance) similar to cultivars from the HIGH group based on results from AC and NA plants (Tables 2.3 and 2.4). This departure in freeze-stress tolerance (i.e., cultivar ranking, Tables 2.3 and 2.4) associated with Pennfine contributed to the significant differences observed among LOW cultivars that was detected in survival (Table 2.1) and EL (Table 2.2). For example, Pennfine AC plants had a mean LT_{50} for survival of -11.8°C and ranked 2nd to LRF-94-C8 (a High cultivar)

in low-temperature survival. Gusta et al. (1980) reported an LT_{50} value of -9.0°C for Pennfine following acclimation. The difference observed in the LT_{50} values point out the difficulties in extrapolating results between experiments because of physiological differences (von Fircks and Verwijst, 1993; Anisko and Lindstrom, 1995). The lack of consistency may be due to a number of physiological differences relating to the stage of development (age) of the plant material, cooling rate, the duration of acclimation and thawing, the method of ice nucleation as well as other factors (Alberdi and Corcuera, 1991).

It is important to recognize that turfgrass quality (the basis for selection of HIGH and LOW groups) is an integration of several quality components including density, color, texture, and uniformity. The loss in shoot density due to direct low-temperature kill (i.e. winter mortality) is only one of many biotic and abiotic stresses operating during the year affecting turfgrass quality. Recent research has shown that improved disease resistance is closely associated with superior turfgrass performance in perennial ryegrass varieties (Ebdon and Gauch, 199_), and Pennfine's poor (low) performance rating in variety trials is in part due to its susceptibility to leaf spot (*Bipolaris* spp.) and brown patch (*Rhizoctonia solani*) disease. Consequently, Pennfine shares some of the attributes in common with high performing cultivars (i.e. superior freeze-stress resistance) important in adapting to northern climates, but its susceptibility to disease is an obvious limitation. Therefore, improved low-temperature survival alone does not necessarily equate to superior turfgrass performance, but rather may be an important consideration in breeding programs along with improved

insect and disease resistance, environmental-stress tolerance, and reduced mowing, fertilizer, and irrigation requirements. Ebdon and Gauch (199_) reported significant genotype by environment interactions in analyzing the 1990 NTEP perennial ryegrass variety trial (1991 through 1994). They found large changes in cultivar rank order from location-to-location in the cool-season turfgrass growing region. Consequently, the differences detected here between top (HIGH) and bottom (LOW) ranked turfgrass performers in low-temperature hardiness (EL, survival) from Maine (Orono NTEP location) may not necessarily hold for a different set of cultivars from locations (environments) with different biotic and abiotic stresses.

Slope estimates from predicted EL (using Eq. 1, shown in Table 2.6) allow for comparison among cultivars based on the 90% confidence interval (CI) from non-linear regression analysis. The CI values shown in Table 2.6 indicated no differences were detected in the curve of the slope among individual cultivars. However, differences were detected between tissue type (AC vs. NA) and between performance type (HIGH vs. LOW). For example, NA plants had a significantly steeper slope (corresponding to 0.63) compared to AC tissue (mean slope corresponding to 0.42). The flatter slope associated with AC crowns compared to NA, indicate that AC plants had reduced EL levels compared to NA tissues. The slope of the EL curve is an important indicator of mortality resulting from freeze stress (Gudleifsson et al., 1986; Zhu and Liu, 1987). The difference in slope between AC and NA tissues and its relationship to mortality is apparent in the EL-survival curves for individual cultivars (Fig. 2.3

to 2.12). For example, it can be seen from the individual EL and survival curves when considered jointly, as freeze-stress temperatures decrease, EL increases with corresponding decrease in survival (increase mortality). This rate of increase is faster (indicated by steeper slope estimates, *B*) for NA plants. A difference in slope implies that acclimation affected the rate of membrane leakage in these cultivars. Differences in slope (from EL curves) between hardened and non-hardened perennial turfgrasses (Rajashekar et al., 1983) and forage grasses (Gudleifsson et al., 1986) suggest that most hardy species have smaller slopes (flatter curves) while non-hardy species are associated with EL curves having steep slopes.

The slope of the curve is generally correlated with the lethal killing temperature of the tissue (Zhu and Liu, 1987). Recall, with AC plants, the HIGH group had significantly higher freeze-stress tolerance (lower LT_{50} values, Table 2.3) based on freeze-shock survival evaluations. Furthermore, the HIGH group had significantly smaller (flatter) slopes ($p \leq 0.10$, Table 2.6, corresponding to 0.39) compared to the LOW group with a mean slope of 0.45. This indicated that AC crowns for the HIGH group had reduced EL compared to their LOW group counterpart. For NA tissue, no difference in survival (LT_{50} value) and slope were observed. Therefore, differences in survival and slope (mortality) between contrasting turf performance groups were detected only after tissues adjusted to low temperature through acclimation.

By tracing a vertical line at the LT_{50} temperature (shown in the EL-survival curves, Fig. 2.3 through 2.12, for AC-survival) until the line intersects the AC-EL

curve, the corresponding EL level at 50% survival can be obtained. This allows comparing cultivar EL at identical survival levels. Using this method, individual cultivar LT_{50} derived from regrowth-survival of AC crowns on average corresponded to an EL level near 77% for the HIGH group (Fig. 2.3 through 2.7), while cultivars from the LOW group (Fig. 2.8 through 2.12) averaged near 60% EL. This difference in EL between groups was significant (at $p \leq 0.10$, data not shown). These results suggest that greater membrane leakage was occurring with the high performance cultivars at equivalent survival levels compared to the LOW group. This leakage level for high performance cultivars may be too high based on the assumption that a leakage level near 50% or more is lethal (Fry et al.,1993). However, Cardona et al. (1997) suggested that intraspecific differences may exist (for certain species) in the amount of irreversible damage to crown tissue. Furthermore, a high leakage level may not always equate to higher membrane damage when plants have been allowed to acclimate to low temperatures (Uemura and Steponkus, 1994; Uemure et al., 1995). For example, Uemura et al, (1995) reports EL levels in *Arabidopsis thaliana* as high as 90% (corresponding to 50% survival). In this present study, leakage levels as high as 88% were observed for the cultivar LRF-94-C8 (Fig. 2.3), a high performance cultivar. Similar EL levels (at 50% survival) for HIGH and LOW groups was observed for NA plants. The HIGH and LOW groups averaged 63 and 65% EL, respectively. Accordingly, the nearly 20% difference in EL at equivalent survival levels reported here between turfgrass quality groups were the results of physiological adjustments associated with acclimation. In

acclimated plants, 50% survival does not necessarily equate to 50% leakage because regrowth survival is a distinctly different phenomenon from leaked electrolytes.

Whole plant survival to freezing temperature depends on tiller production in bunch type grasses such as perennial ryegrass. Improved survival of hardened perennial ryegrass to lower freezing temperatures is depended on the ability for regrowth from lateral tiller buds during recovery, and is not attributed to improved hardness of the main apex (Eagles et al., 1993). Furthermore, the growth apex of the main tiller of perennial ryegrass is killed at higher temperatures than other regions of the crown. Thus, differential hardening of perennial ryegrass crown tissues may be occurring. Consequently, high leakage rates can occur from crown tissues and regrowth may still be possible providing critical crown regions remain viable. In orchardgrass (*Dactylis glomerata* L.) Shibata and Shimada (1986) also observed greater damage in the main shoot apices than in lateral tiller buds. This type of differential hardening observed between species may also occur between cultivars, however, differential hardening studies at the intraspecific level have not been conducted. Differential hardening has also been identified in winter wheat crowns (Tanino and McKersie, 1985) where survival to freezing stress was limited by the tolerance of a relatively small number of cells associated with the vascular transition zone in the basal region. Similarly, the lower portion of annual bluegrass (*Poa annua* L.) crowns was found to be more susceptible to freezing injury than the upper region (Beard and Olien, 1963).

Eagles et al. (1993) suggested that the release of apical dominance after death of the main apex was the method of recovery in perennial ryegrass. They also reasoned that profuse tillering could have a beneficial effect on recovery by providing more tiller buds as potential sites for regrowth. In this present study, no tiller counts were made to allow comparing the relative tillering capacities of HIGH and LOW groups. However, shoot density measurements obtained from NTEP reports (USDA, 1997) indicate that the high performance cultivars had significantly more shoots (based on field observations) compared to the LOW group, and this implies the potential for greater tillering and, therefore, winter survival with the high performance cultivars as suggested by Eagles et al. (1993).

Figure 2.13 (showing AC tissues) and 2.14 (showing NA tissues) reveal differences in EL and survival comparing HIGH and LOW groups. The scatter points shown in these figures are the corresponding mean response (EL or survival) of individual cultivars from the HIGH group (Fig. 2.3 through 2.7) and LOW group (Fig. 2.8 through 2.12) by tissue type (AC and NA) and temperature (+5 through -21°C). The lethal temperature (LT_{50}) were computed mathematically as previously described for individual cultivars. There was good agreement between the LT_{50} estimates derived directly from the EL-survival curves corresponding to HIGH and LOW groups (Fig. 2.13 and 2.14) with the mean of the LT_{50} estimates derived from the individual cultivar EL-survival curves shown in Tables 2.3 and 2.4. The exception to this was AC-survival for the LOW group (Fig. 2.13). Specifically, the $LT_{50\ LOW}$ estimate for AC-survival

was -7.3°C while the mean of the individual LT_{50} estimate derived from survival curves corresponding to the five LOW cultivars was -6.0°C (Table 2.3). This discrepancy of 1.3°C however, was due to Linn and SR-4010 cultivars which were arbitrarily given LT_{50} estimates of -3.0°C (Table 2.3) because they did not survive the AC conditions (i.e., -14.8°C minimum air temperature and -12.6°C minimum media surface temperature, Fig. 2.1 and 2.2 respectively).

Based on freeze-shock survival evaluations, the HIGH group had significantly higher survival ratings compared to the LOW group for both AC and NA tissues. For AC plants, HIGH cultivars were associated with greater survival compared to the LOW group at freeze-stress temperatures as low as -13°C (Fig. 2.13). Even at the non-frozen control temperature (+5°C) the HIGH cultivars afforded significantly higher survival compared to the LOW group. This most likely was due to the low temperatures associated with the AC treatment in January that may have approached the critical lethal thresholds for some of the LOW cultivars. However, this does not explain the superior survival ratings with the HIGH group for NA tissue at the non-frozen control temperature (Fig. 2.14). For example, the HIGH group had a mean survival of 80.5% while the LOW group had a mean survival of 64%, a 16.5% greater survival rating associated with the HIGH group. It is important to recognize however, that the difference in survival between the two groups increased significantly ($p \leq 0.10$) by another 10.5% at -3°C when compared to the control temperature, indicating superior survival with the HIGH group. No difference in survival for NA plants was

detected between the two performance groups at freeze-stress temperatures lower than -5°C.

Similarly, the HIGH group also had significantly less leakage loss (hence, less membrane damage) compared to the LOW cultivars. However, group differences in EL were detected over a small range of freeze-stress temperatures compared to regrowth-survival evaluations. For example, with AC plants, significantly lower EL was detected with HIGH cultivars only down to -7°C (compared to -13°C for survival), while with NA tissue less leakage was observed with the high performance cultivars only to a temperature of -3°C (compared to -5°C for survival). Like NA-survival evaluations, differences between groups were detected for NA-EL at the non-frozen control temperature. The high group had a mean EL of 25.3% compared to 33.6% for the LOW group, or equivalently 8.3% less leakage with the high performance cultivars. However, differences in EL increased significantly (at $p \leq 0.001$) by another 8.1% at -3°C when compared to the non-frozen control indicating superior low-temperature tolerance for the HIGH group.

Significant acclimation and cultivar effects of perennial ryegrass occurred. Perennial ryegrass cultivars that consistently performed well (ranking in the top 5%) in turfgrass variety trials conducted at the Maine-Orono NTEP location (most northern NTEP location in New England) shared similar freeze-stress characteristics in common and were different from their poor performing counterparts (ranking in the bottom 5%). Compared to low performance cultivars, high performance cultivars exhibited higher freeze-stress survival, less

membrane leakage with EL curves having smaller slopes (lower mortality), and lower LT_{50} values based on EL and regrowth-survival methods. The ultimate goal of a turfgrass breeding program is to develop cultivars that are well adapted to a wide range of management and environmental stresses. In perennial ryegrass, winter hardiness is a heritable trait (Waldron et al., 1998), suggesting that the potential exists to develop improved perennial ryegrass cultivars with increased low-temperature tolerance through breeding. This study indicated that improved low-temperature tolerance is an important selection criteria for developing perennial ryegrass with superior quality when targeting genotypes to northern climates. As a point of reference, the January air temperature for Maine averaged -10.3°C during the period of evaluation on which the cultivar selections used in this study were based. Other cool-season turfgrass locations with winter conditions similar to Maine may also find a strong relationship between turfgrass performance and low-temperature tolerance, and therefore, may also benefit from low-temperature assessment studies. To that end, screening for superior low temperature survival using electrolyte leakage methods may be useful along with whole-plant regrowth-survival evaluations (a more reliable indicator of plant winter hardiness) and where ranking of cultivars is the goal. In these studies, cultivar LT_{50} values based on EL evaluations were comparatively narrow, while cultivar survival was variable (broad) thus allowing greater potential to develop screening protocols.

High membrane leakage rates in these studies did not necessarily equate to permanent injury as indicated by total death of the tissue (crown). In fact,

cultivars representative of the highest in turfgrass quality exhibited higher leakage levels than poor performing cultivars when compared at equivalent survival levels. Further studies are required to investigate the specific mechanism allowing differential hardening during acclimation within the crown region (main apical meristem, lateral tiller buds, and transitional zone) between these two contrasting performance groups. Specific research needed at the intraspecific level in perennial ryegrass includes; (i) the pattern of recovery by crown region after artificial freezing as suggested by Eagles et al. (1993), (ii) microscopic examinations for freezing-stress injury in the crown region which have been useful at the interspecific level (Beard and Olien, 1963) and may be needed at the genotypic level, and (iii) vital staining techniques (Tanino and McKersie, 1985) of crown tissue after artificial freezing to identify critical regions important in regrowth survival.

Table 2.1 Mean squares from ANOVA of freeze-shock recovery (survival) of ten perennial ryegrass cultivars representing high and low performance types evaluated at 11 freeze-stress temperatures and two environments (acclimated and non-acclimated).

Source of variation	df	MS
Total	879	
Block	3	6131.6***
Cultivar (C)	9	3871.7***
High vs. low	1	18456.0***
Among high performers	4	316.3
Among low performers	4	3781.3***
Temperature (T)	10	34439.3***
Environment (E)	1	143310.1***
C x T	90	365.3
C x E	9	2708.6***
T x E	10	8280.9***
C x T x E	90	408.0
Error	657	387.6

*** Significant at the 0.001 level.

Table 2.2 Mean squares from ANOVA of crown electrolyte leakage of ten perennial ryegrass cultivars representing high and low performance types evaluated at 11 freeze-stress temperatures and two environments (acclimated and non-acclimated).

Source of variation	df	MS
Total	659	
Block	2	235.4**
Cultivar (C)	9	346.8***
High vs. low	1	1742.3***
Among high performers	4	126.7*
Among low performers	4	218.0***
Temperature (T)	10	26104.7***
Environment (E)	1	38218.2***
C x T	90	51.2†
C x E	9	136.8***
T x E	10	3372.0***
C x T x E	90	43.0
Error	438	42.2

†, *, **, *** Significant at the 0.1, 0.05, 0.01, and 0.001 levels, respectively.

Table 2.3 Comparison of LT_{50} estimates from fitted crown freeze-shock survival curves.

	LT_{50} by Environment			
Perennial Ryegrass Cultivar	Acclimated (AC)		Non-Acclimated (NA)	
High Performance Group	Rank	LT_{50}	LT_{50}	Rank
LRF-94-C8	1	-14.7[†]	-3.7	2
Palmer III	5	-10.0	-2.9	3
Prelude III	6	-9.3	-4.2	1
RepeII III	3	-10.2	-2.7	4.5
Top Hat	4	-10.1	-1.8	7
Mean-High Group	-	-10.9	-3.0	-
Low Performance Group				
DSV NA 9401	8	-3.9	-2.2	6
DSV NA 9402	7	-8.3	-1.2	8
Linn	9.5	-3.0	3.9	10
Pennfine	2	-11.8	-2.7	4.5
SR-4010	9.5	-3.0	-1.1	9
Mean-Low Group	-	-6.0	-0.7	-
Grand Mean (by Environment)	-	-8.4	-1.8	-
Significance (*p*-value)				
Mean High vs. Low[‡]	-	0.05	NS	-
Mean AC vs. NA[§]	-	0.001		-

[†] Lethal temperature in °C.
[‡] Comparison between group means within environment (AC and NA).
[§] Comparison between environment means.

Table 2.4 Comparison of LT_{50} estimates from fitted crown electrolyte leakage curves.

	LT_{50} by Environment			
Perennial Ryegrass Cultivar	Acclimated (AC)		Non-Acclimated (NA)	
High Performance Group	Rank	LT_{50}	LT_{50}	Rank
LRF-94-C8	1	-6.7[†]	-0.8	5
Palmer III	2	-6.0	-2.1	3
Prelude III	6	-5.2	-2.2	2
Repell III	5	-5.5	-2.6	1
Top Hat	8	-3.4	-1.4	4
Mean-High Group	-	-5.3	-1.8	-
Low Performance Group				
DSV NA 9401	7	-4.7	0.1	6
DSV NA 9402	4	-5.6	0.3	7
Linn	9	-1.2	0.4	8
Pennfine	3	-5.8	1.0	9
SR-4010	10	-0.5	1.6	10
Mean-Low Group	-	-3.6	0.7	-
Grand Mean (by Environment)	-	-4.4	-0.6	-
Significance (*p*-value)				
Mean High vs. Low[‡]	-	0.1	0.05	-
Mean AC vs. NA[§]	-	0.001		-

[†] Lethal temperature in °C.
[‡] Comparison between group means within environment (AC and NA).
[§] Comparison between environment means.

Table 2.5 Correlation between 10 perennial ryegrass cultivar LT_{50s} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations using acclimated (AC) and non-acclimated (NA) tissue.

	EL		Survival	
	AC	NA	AC	NA
EL-NA	0.50	-	-	-
Survival-AC	0.80**	0.48	-	-
Survival-NA	0.72*	0.46	0.66*	-

*, **, Significant at the 0.05 and 0.01 levels, respectively.

Table 2.6 Comparison of slope estimates from fitted crown electrolyte leakage curves.

Perennial Ryegrass	Slopes Estimate ±90% CI† by Environment	
High Performance Group	Acclimated (AC)	Non-Acclimated (NA)
LRF-94-C8	0.38±0.21	0.64±1.01
Palmer III	0.42±0.22	0.69±0.81
Prelude III	0.41±0.22	0.49±0.33
Repell III	0.40±0.24	0.81±1.38
Top Hat	0.36±0.28	0.68±0.60
Mean-High Group	0.39	0.66
Low Performance Group		
DSV NA 9401	0.50±0.29	0.52±0.47
DSV NA 9402	0.35±0.13	0.56±0.23
Linn	0.43±0.25	0.74±2.17
Pennfine	0.54±0.33	0.72±0.66
SR-4010	0.45±0.31	0.40±0.29
Mean-Low Group	0.45	0.59
Grand Mean (by Environment)	0.42	0.63
Significance (*p*-value)		
Mean High vs. Low‡	0.1	NS
Mean AC vs. NA§	0.001	

† No difference in slope between individual cultivars and environments based on 90% confidence intervals (CI).

‡ Comparison between group means within environment (AC and NA).

§ Comparison between environment means.

NS = not significant.

Fig. 2.1. Mean daily air temperature comparison between acclimated (polyhouse) and non-acclimated (greenhouse) environments during the experimental period.

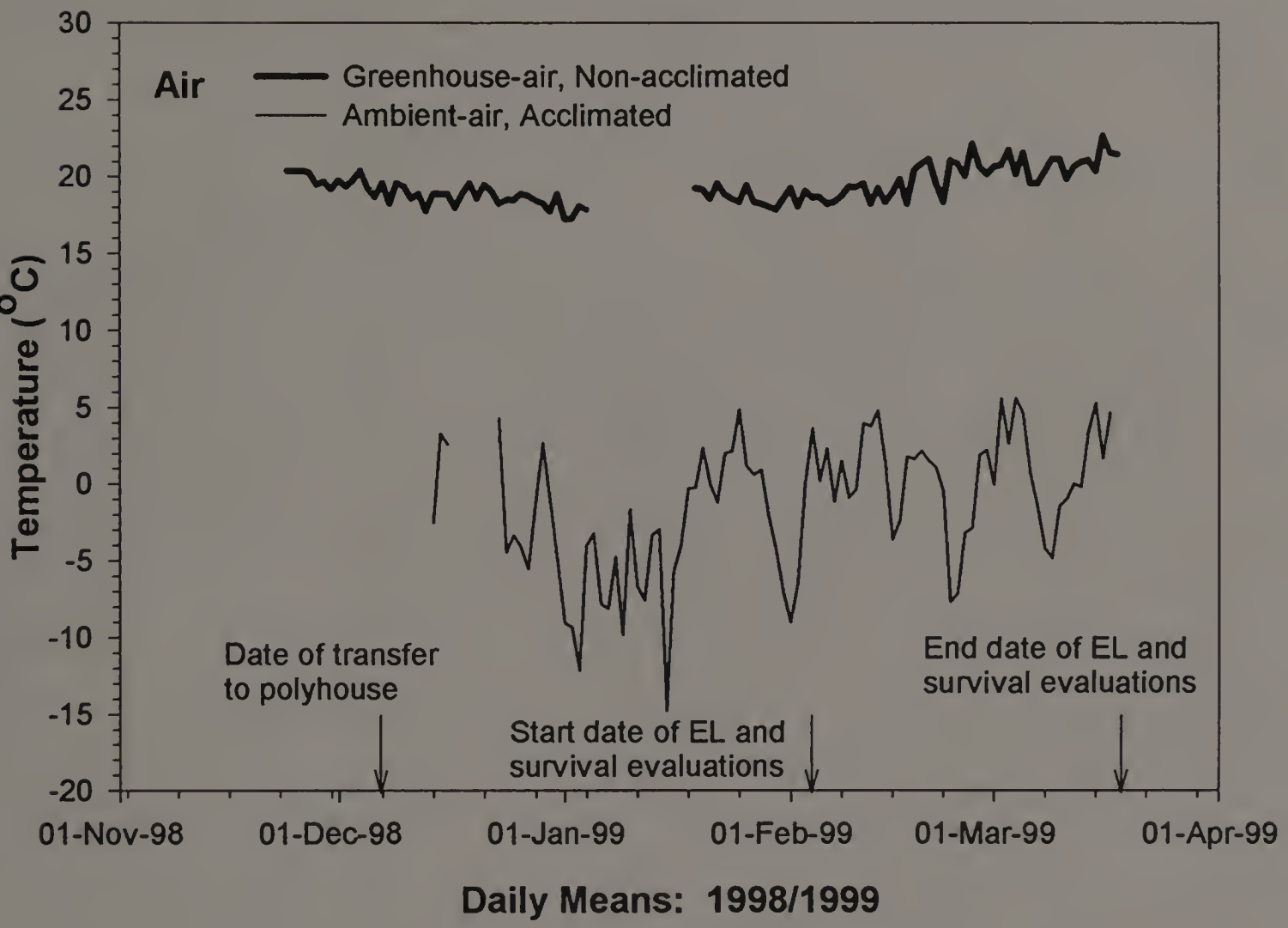


Fig. 2.2. Mean daily soil temperature comparison between actual soil and plant container media at various depths during the experimental period.

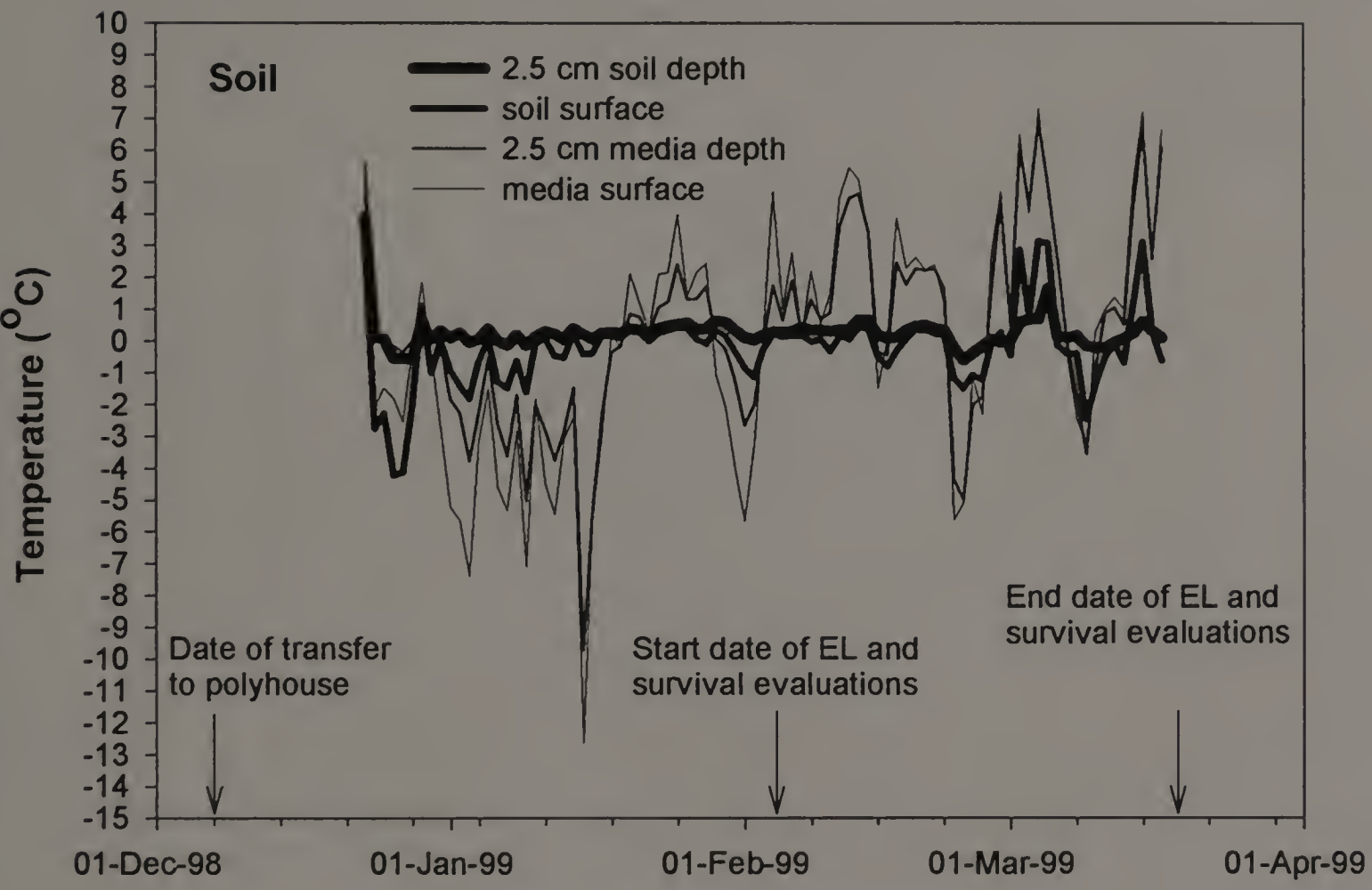


Fig. 2.3. LRF-94-C8 perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

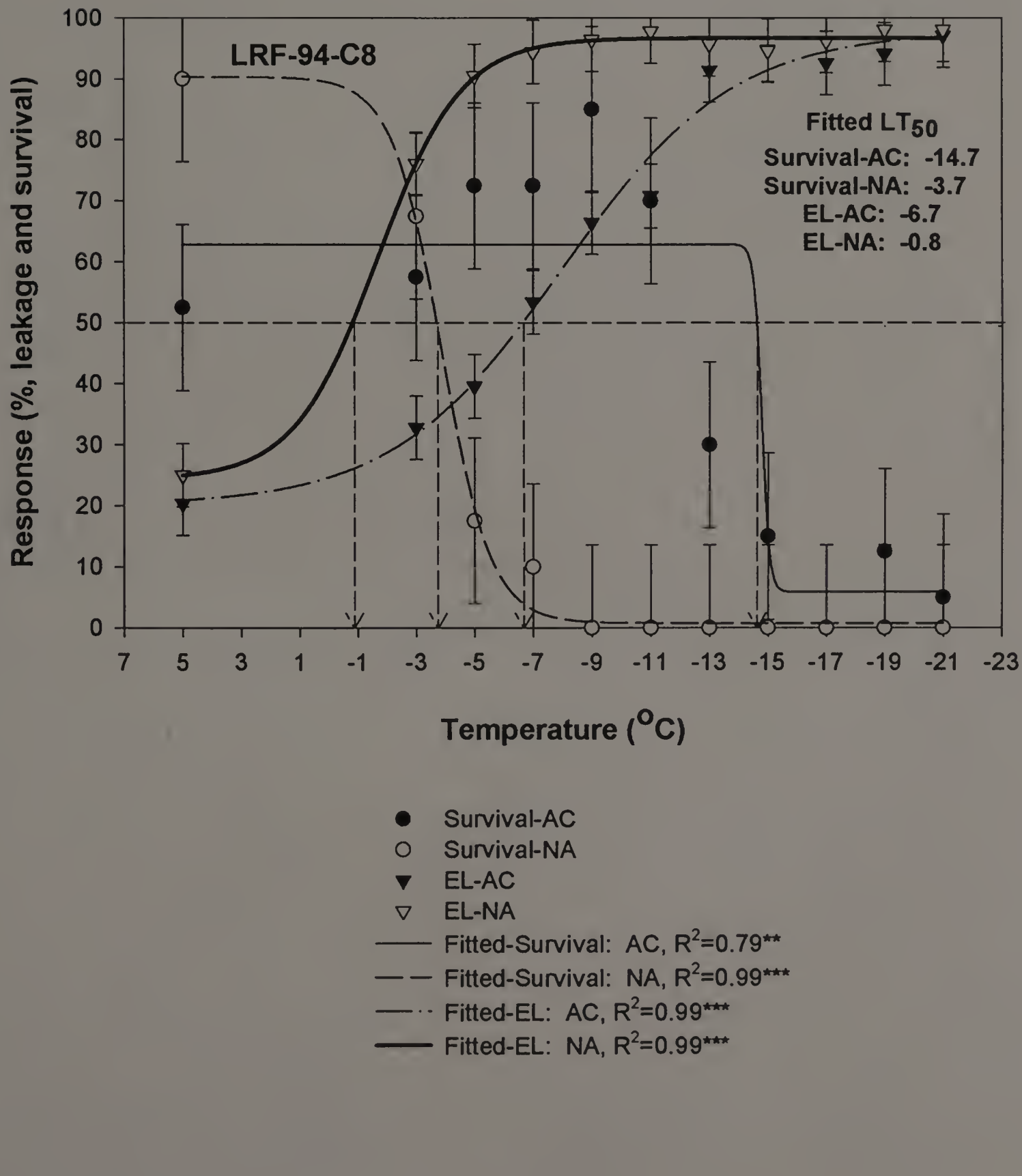


Fig. 2.4. Palmer III perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

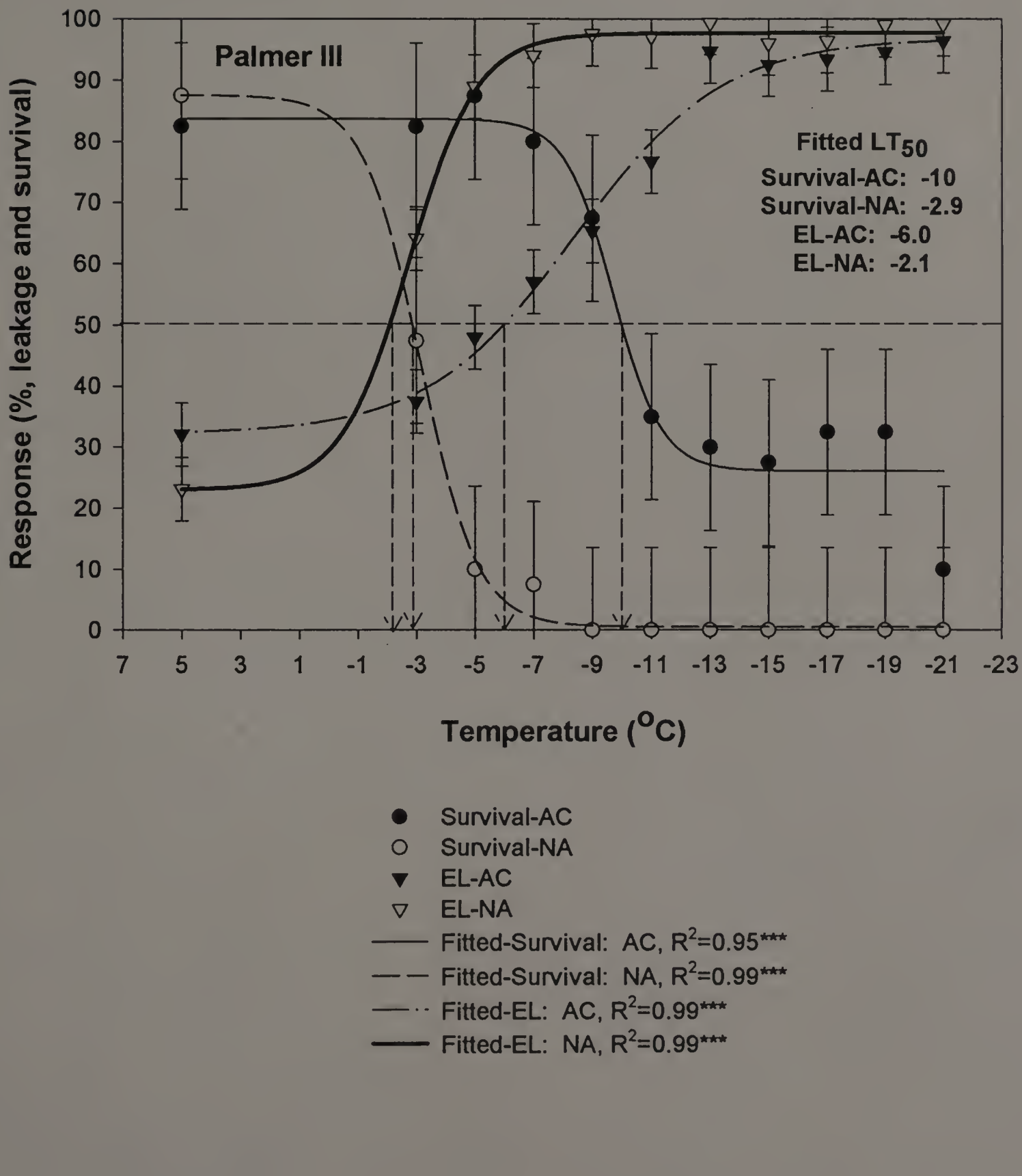


Fig. 2.5. Prelude III perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

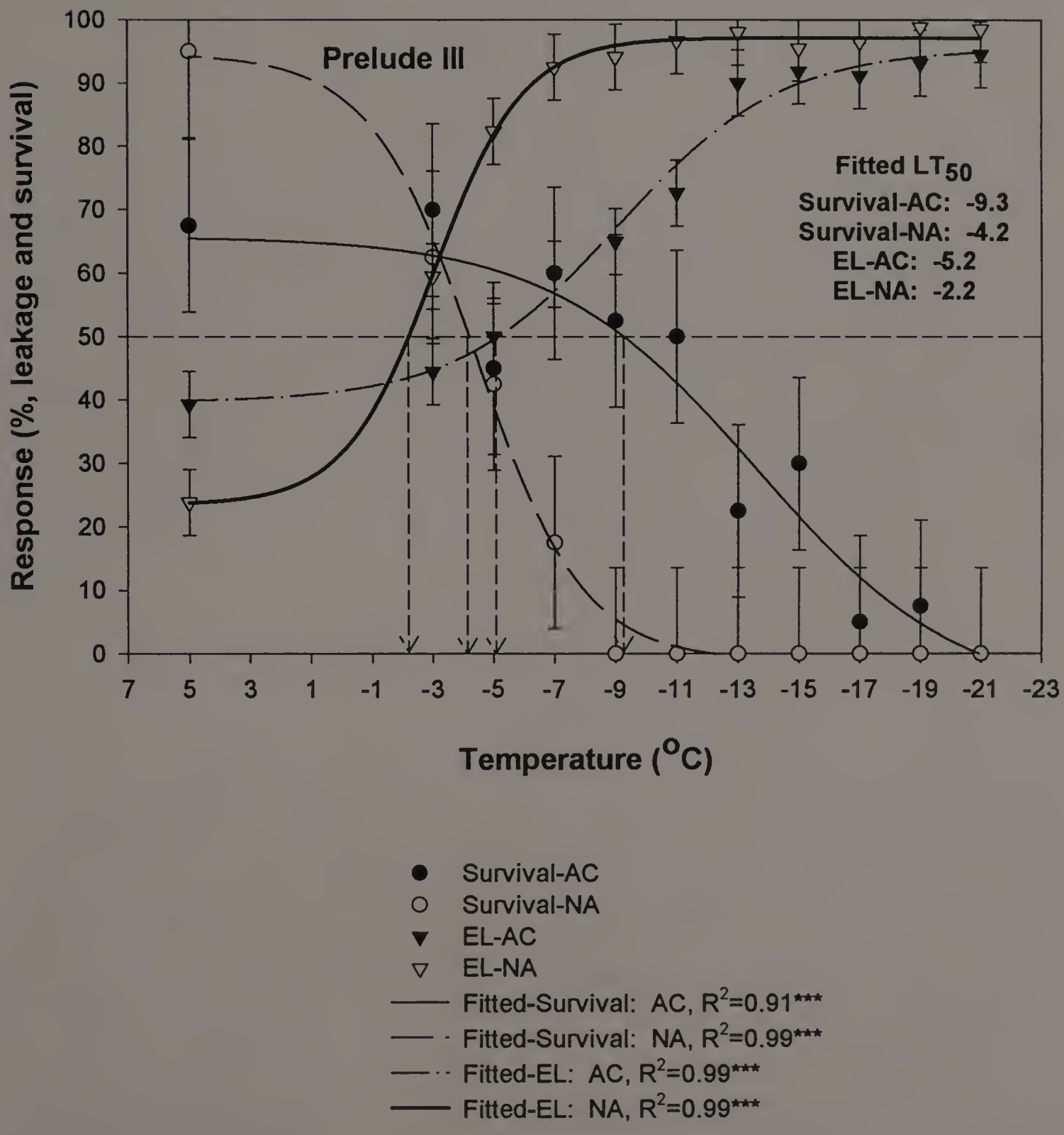


Fig. 2.6. Repell III perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

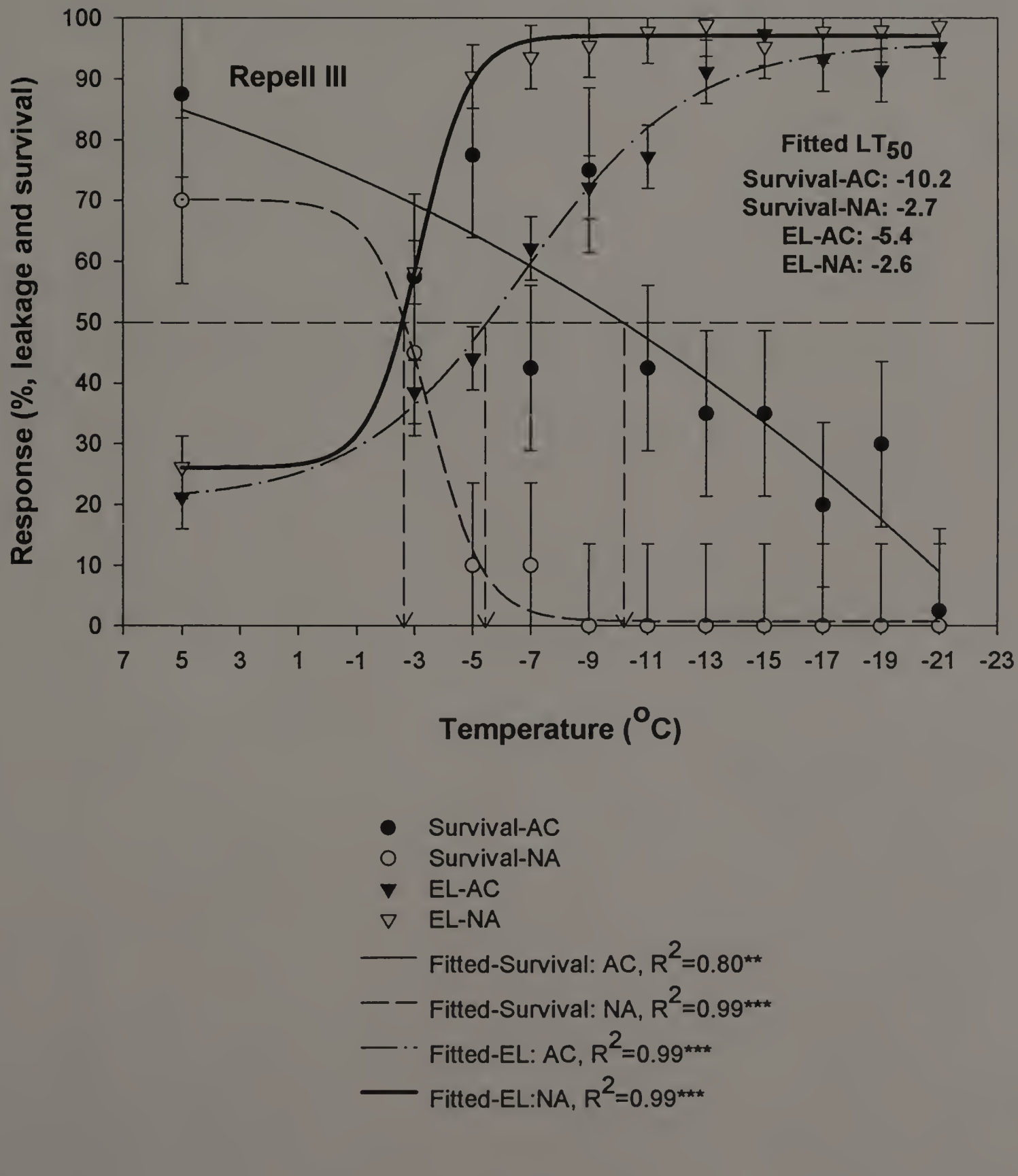


Fig. 2.7. Top Hat perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

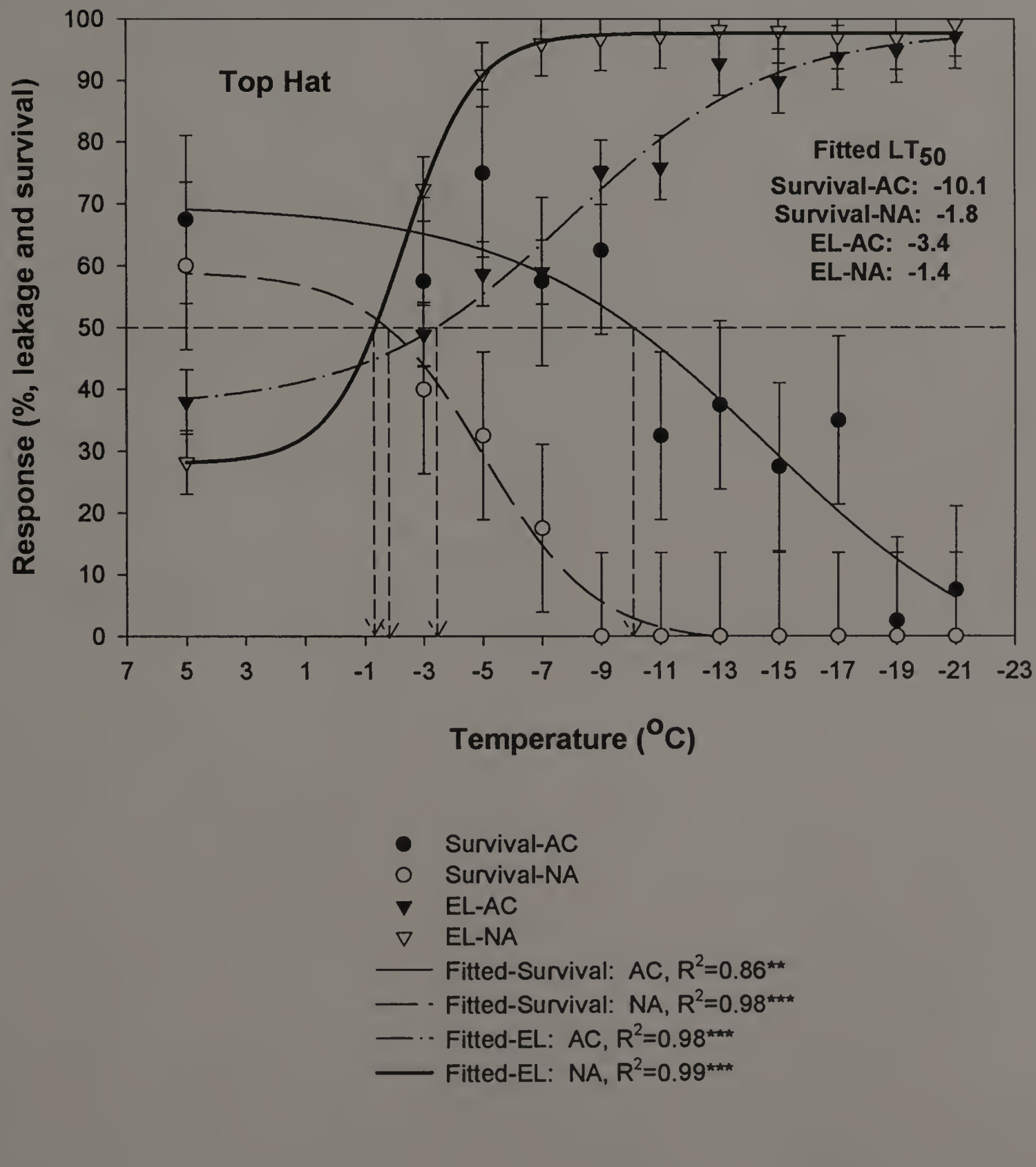


Fig. 2.8. DSV-NA-9401 perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

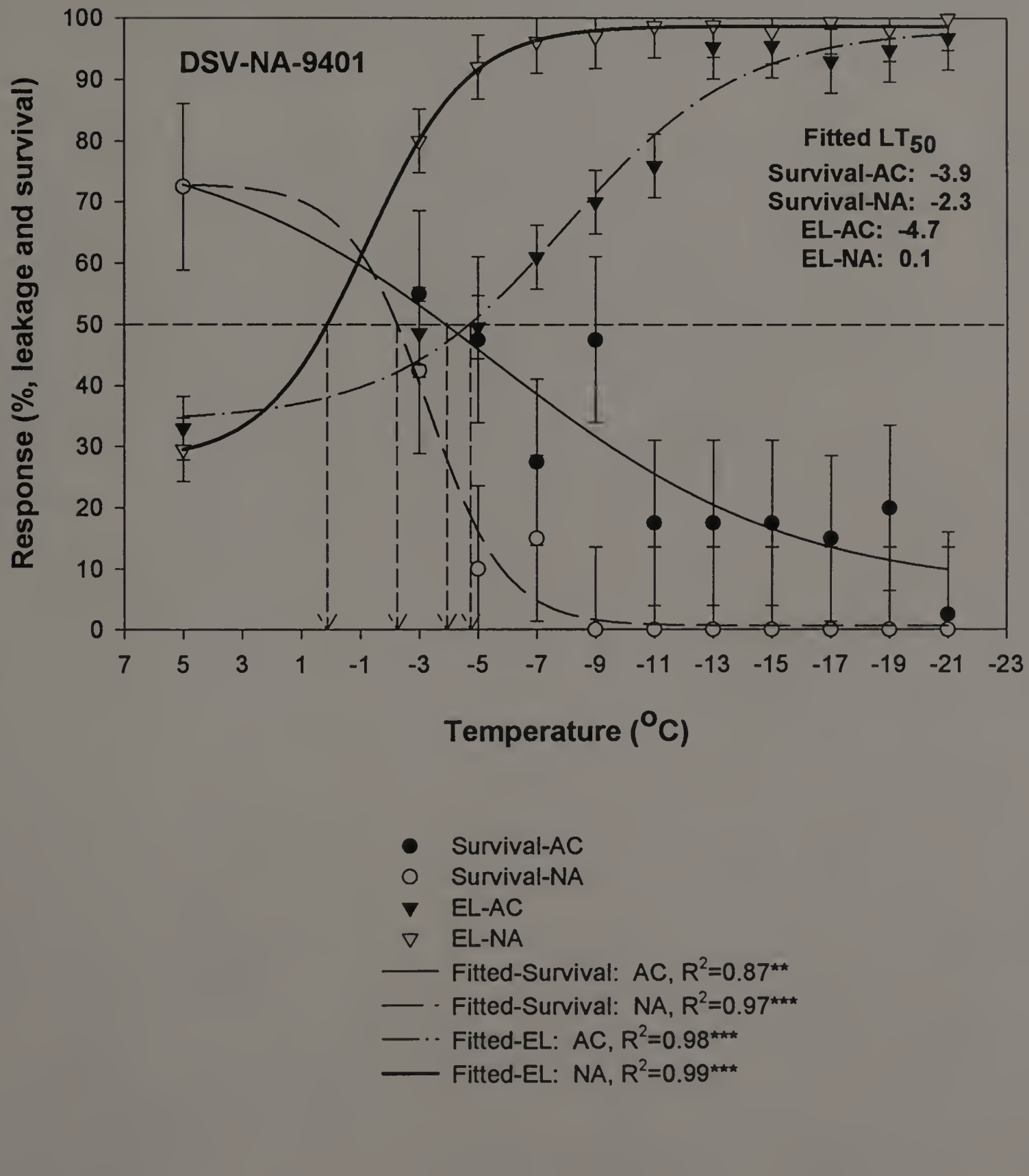


Fig. 2.9. DSV-NA-9402 perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within response (EL, survival).

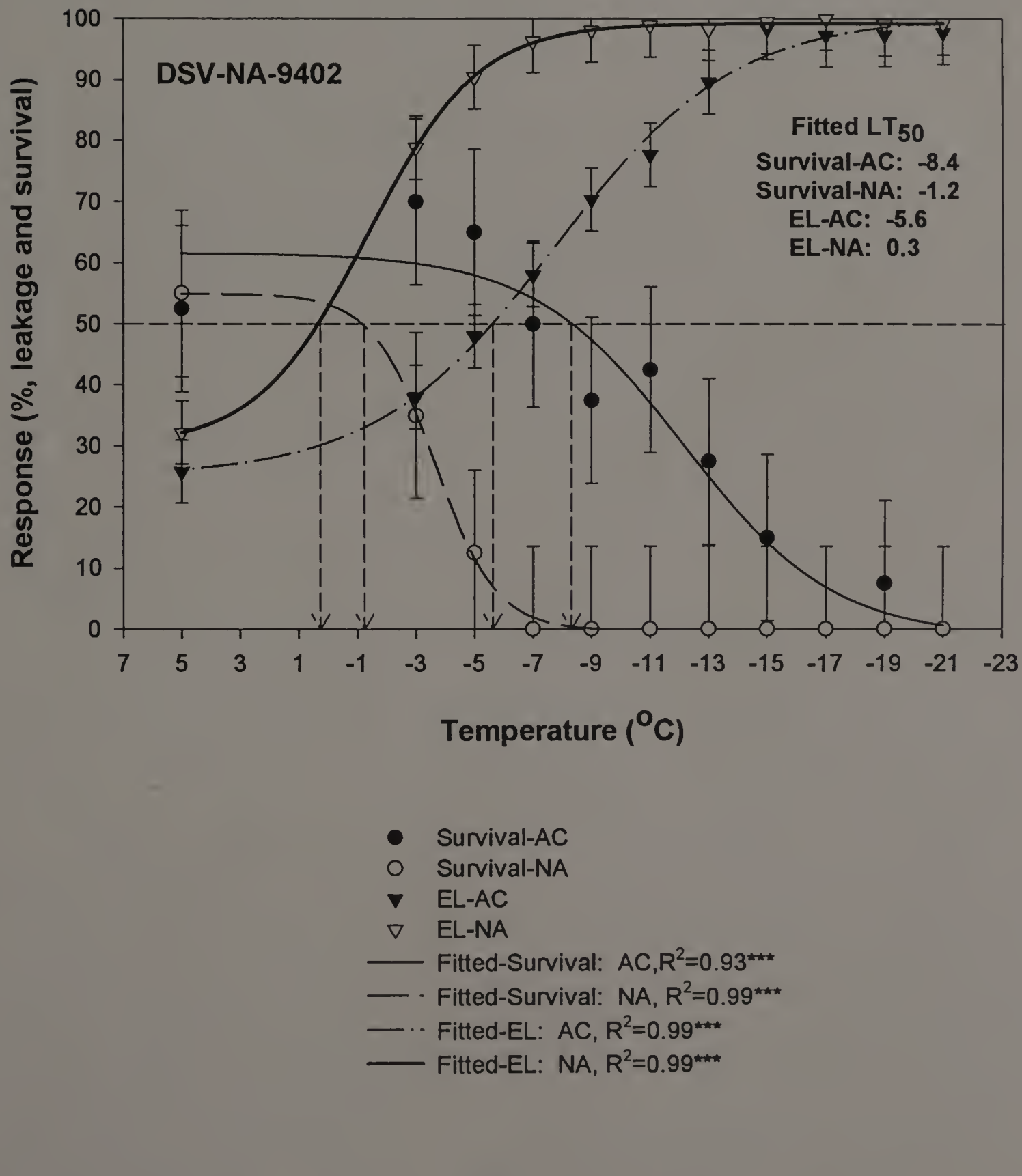


Fig. 2.10. Linn perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within responses (EL, survival).

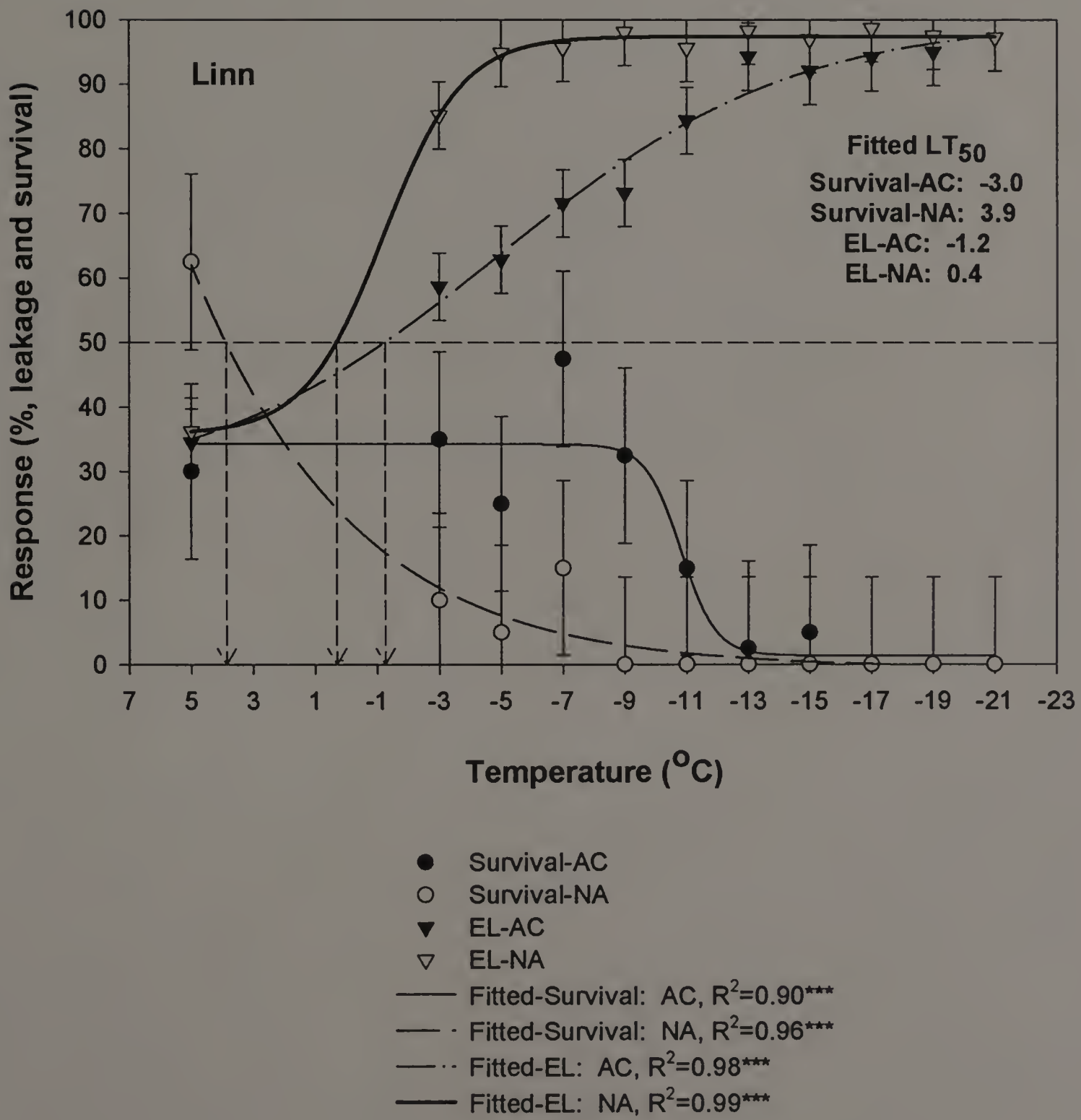


Fig. 2.11. Pennfine perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within responses (EL, survival).

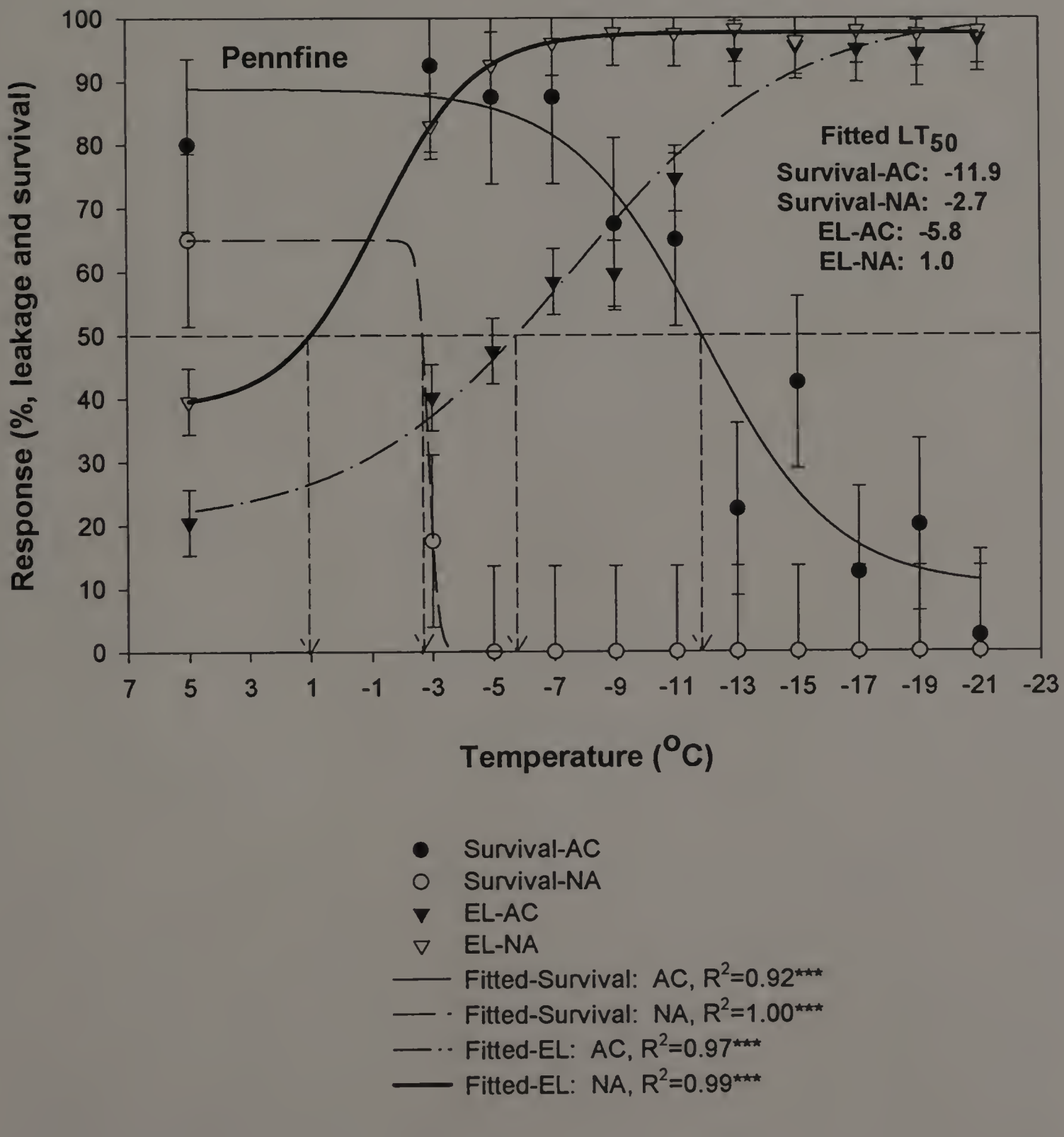


Fig. 2.12. SR-4010 perennial ryegrass fitted LT_{50} derived from crown electrolyte leakage (EL) and freeze-shock survival evaluations for acclimated (AC) and non-acclimated (NA) tissues. LSD (0.05) bars are shown for comparison within responses (EL, survival).

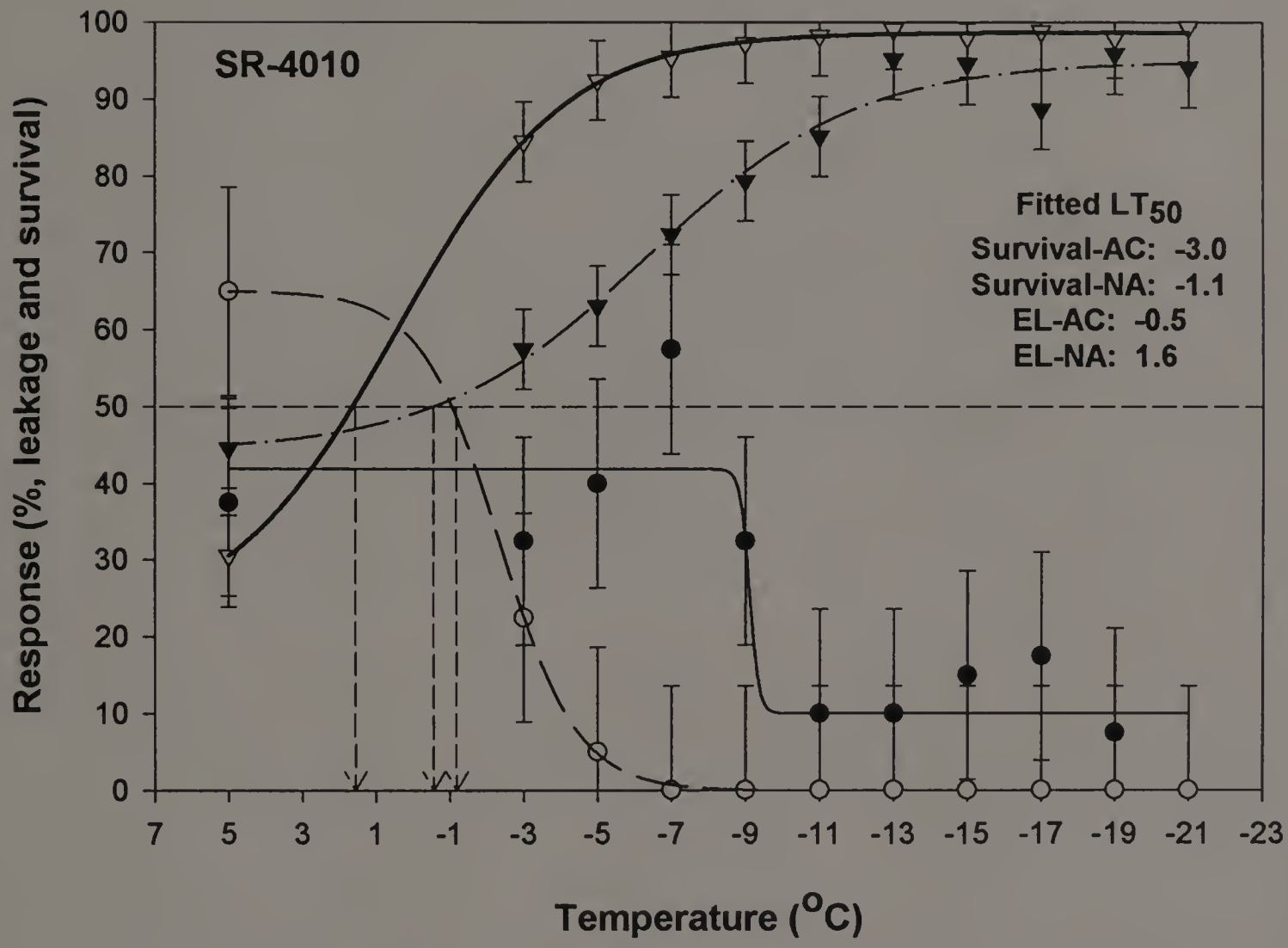


Fig. 2.13 Fitted LT_{50} derived from acclimated crown electrolyte leakage (EL) and freeze-shock survival evaluations for high and low performance cultivars of perennial ryegrass. LSD (0.05) bars are shown for comparison within response (EL, survival).

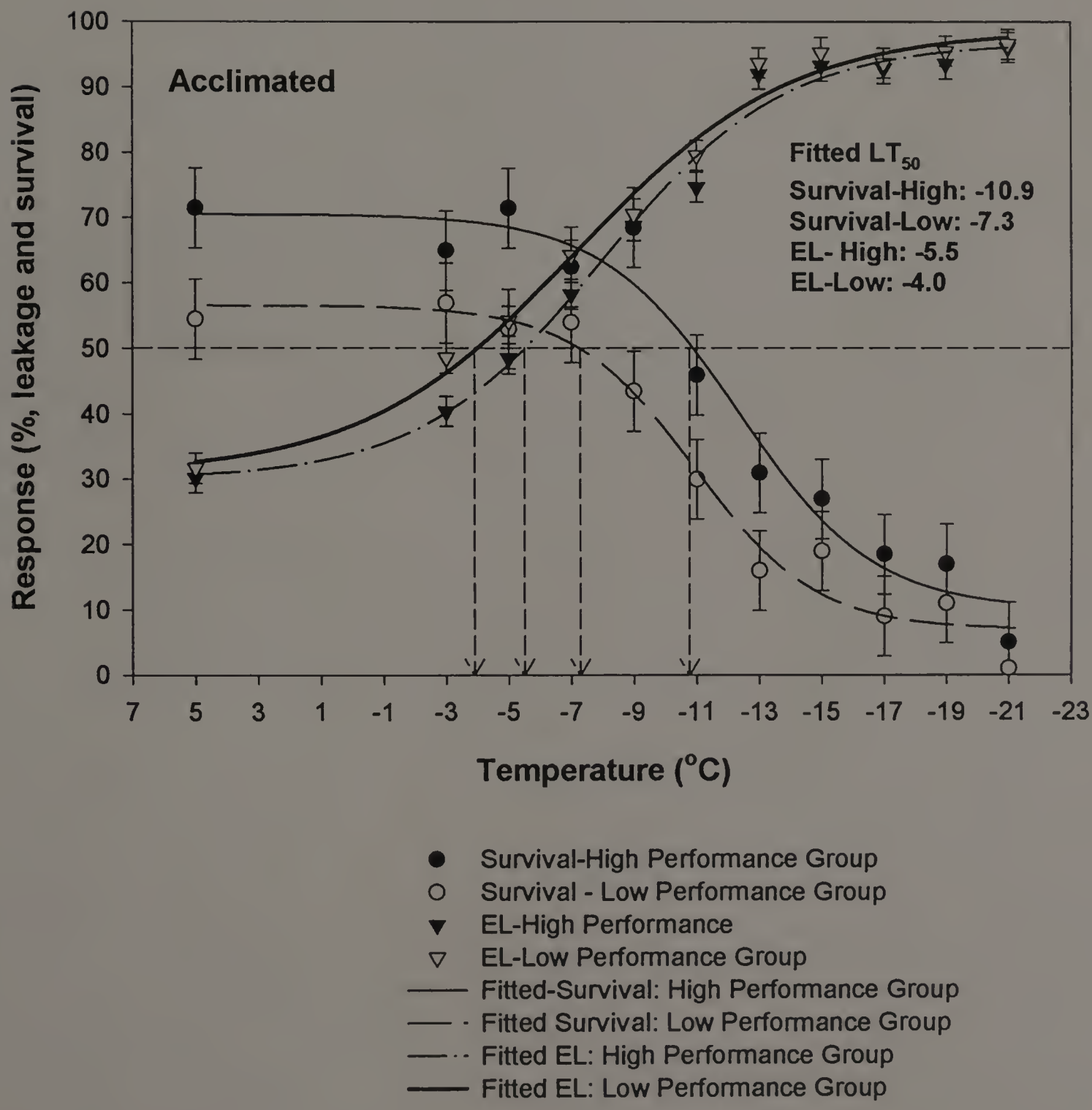
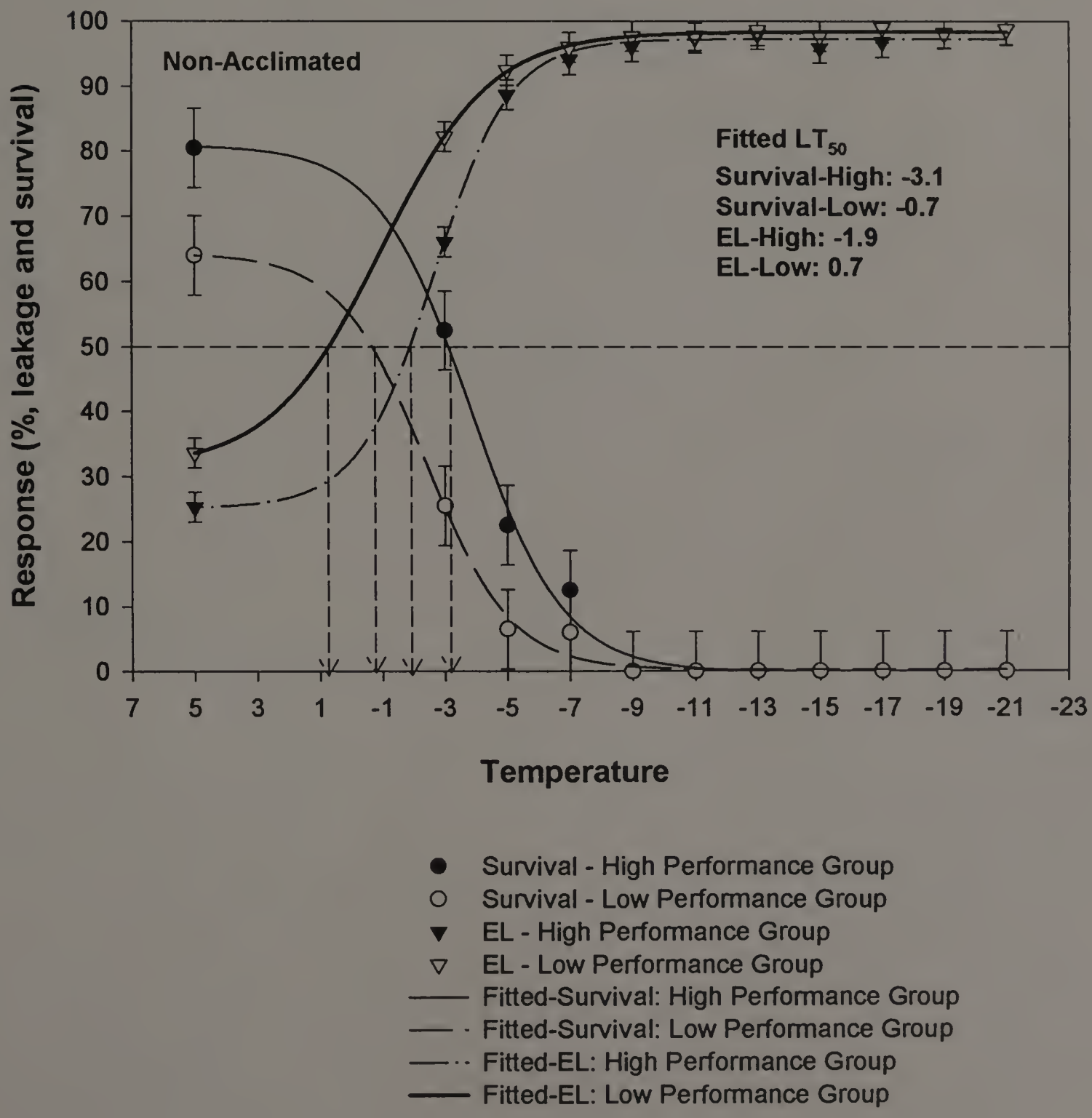


Fig. 2.14 Fitted LT_{50} derived from non-acclimated crown electrolyte leakage (EL) and freeze-shock survival evaluations for high and low performance cultivars of perennial ryegrass. LSD (0.05) bars are shown for comparison within response (EL, survival).



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APPENDIX A

LOCATION OF THERMOCOUPLES

One thermocouple was positioned 25-30 cm above the containers and centered in the cold frame in order to measure air temperature over the turf. Two other thermocouples were positioned at container soil level at approximately the one quarter and three quarter positions in the cold frame in order to measure the temperature of representative container soil surfaces near the turf crowns. Two other thermocouples, used to measure root-zone temperatures, were inserted approximately 5 cm into the soil of two containers at approximately the one quarter and three quarter positions in the cold frame. One thermocouple was inserted approximately 5 cm into the surrounding ground outside the poly house but near the cold frame and a last thermocouple was placed on top of the ground, in the same area, to measure surrounding soil temperatures.

APPENDIX B

FREEZE-SHOCK RECOVERY (SURVIVAL) EVALUATION PROTOCOL

Roger Gagne - University of Massachusetts 24 January 1999

Freezing Tolerance Evaluation of Contrasting Perennial Ryegrass (*Lolium perenne* L.) Performance Groups Using Freeze-Shock Recovery (PRYEFRZSR1)

Objective:

To test the freezing tolerance characteristics, under acclimated and non-acclimated conditions, of 5 genotypes representing high performance perennial ryegrass and 5 genotypes representing low performance perennial ryegrass based on turfgrass performance and quality results obtained from the Maine (Orono) National Turfgrass Evaluation Program (NTEP) location.

Experimental material:

- Acclimated and non-acclimated cultivars representing high performance perennial ryegrass:
  - Repell III
  - Prelude III
  - Palmer III
  - Top Hat
  - LRF-94-C8

- Acclimated and non-acclimated cultivars representing low performance perennial ryegrass:
  - Linn
  - DSV NA 9402
  - DSV NA 9401
  - Pennfine
  - SR-4010 (SRX 4010)

- 220 poly freezer bags for plant samples.

- 5 poly freezer bags for thermocouples.

Acclimated plant material was maintained in containers (5 cm in diam. by 17.8 cm deep) in a cool greenhouse (55-60°F) from 19 Oct 1998 to 9 Dec 1998. On 9 December, the material was transferred to the South Deerfield farm and placed in an unheated (poly) hoop house with the ends opened for air circulation. Plant

containers were placed on the floor of the hoop house in blocks by cultivar and all wrapped together with Corning 3.5" house insulation (R13).

Non-acclimated plant material was maintained in containers 5 cm in diam. by 17.8 cm deep in a greenhouse (61-68 ° F). Containers were placed on a wire shelf, approximately 50 cm off the floor, in blocks by cultivar.

Test temperatures: Non-frozen control (+5°C), -3, -5, -7, -9, -11, -13,-15, -17, -19, and -21°C.

Replication: 10 plants per replicate container

Protocol:

Pre Day 1 (preparation):

1. Label 220 poly freezer bags for plant material using the following code:

2 environments	A - acclimated N - non-acclimated
10 cultivars	C1-C10
11 temperatures	Non-frozen control (+5°C), -3, -5, -7, -9, -11, -13,-15, -17, -19, -21°C

Example: Acclimated, cultivar C1, +5°C

A	C1	+5°

2. Consecutively number 5 poly freezer bags for thermocouples.

Day 1 (Harvesting of material & Preparation of samples):

Harvesting of material:

1. Harvest 12 containers (includes 1 extra if needed) of each cultivar of acclimated material and 12 containers (includes 1 extra if needed) of each cultivar of non-acclimated material.

2. Starting with the acclimated material (handle one container at a time), wash soil from roots of turf material in cold tap water, wrap in moist toweling, place in properly labeled zip-lock bag (leave unzipped), and refrigerate (+5°C). Repeat using the non-acclimated material.

Preparation of samples:

1. Starting with acclimated material (handle one zip-lock bag at a time), separate material into individual plants, trim shoots and roots of 10 plants to 2 cm each, wrap in moist paper toweling, and place in properly labeled poly freezer bags. Close bags and place temporarily in refrigerator. Complete preparation of all acclimated material.

2. When all acclimated material has been prepared, place the bags labeled -3 through -21 in the freezer at - 2°C. Leave the non-frozen control samples, labeled +5, in the refrigerator (+5°C).

Day 2 (continue Preparation of samples):

3. Repeat step 1 and 2 using non-acclimated material. Complete preparation of all non-acclimated material.

4. From excess plant material, wrap 5 groups of 10 plants in moist toweling and add one group to each of the 5 freezer bags marked for the thermocouples.

5. Place a properly marked thermocouple in each bag (making contact with the plant material) and randomly place the bags among the samples.

6. Leave samples overnight with freezer set-point at -2°C.

Day 3 (begin freezing schedule):

1. Visually examine tissue samples to ensure freezing has occurred. Lower freezer set-point to the first scheduled test temperature (-3°C). Once tissue temperature has reached -3°C, as measured by the thermocouples, maintain sample temperature for one hour.

2. Remove all -3 samples (20 bags) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.

3. Lower freezer set-point to the next scheduled test temperature (-5°C). Allow tissue temperature to reach -5°C, as measured by the thermocouples, and maintain sample temperature for one hour.

4. Remove all -5 samples (20 bags) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.

5. Repeat steps 3 and 4 until all scheduled test temperatures up to and including -11°C have been completed. Leave freezer set at -11°C overnight.

Day 4 (continue freezing schedule, begin Freeze-Shock Recovery Evaluation):

6. Lower freezer set-point to the next scheduled test temperature (-13°C). Allow tissue temperature to reach -13°C, as measured by the thermocouples, and maintain sample temperature for one hour.

7. Remove all -13 samples (20 bags) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.

8. Repeat steps 6 and 7 until the remaining scheduled test temperatures have been completed.

Freeze-Shock Recovery Evaluation:

1. Unwrap the non-frozen control samples and the samples thawed overnight from Day 3 freezing (step 5). Re-pot each group of 10 plants in individual cells of 72 cell trays, carefully labeling each cell, and place in the greenhouse (61-68°F) for a 3 to 4 week recovery period (survival evaluation).

Day 5 (continue Freeze-Shock Recovery Evaluation):

2. Unwrap the samples thawed overnight from Day 4 freezing (step 8). Re-pot each group of 10 plants in individual cells of 72 cell trays, carefully labeling each cell, and place in the greenhouse (61-68°F) for a 3 to 4 week recovery period (survival evaluation).

3. Plants that recover will be scored as survivors, all others will be scored as having been killed by the temperature treatment.

% Survival = (number of plants survived / total number of plants) x 100

APPENDIX C

ELECTROLYTE LEAKAGE ASSAY PROTOCOL

Roger Gagne - University of Massachusetts 9 February 1999

Freezing Tolerance Evaluation of Contrasting Perennial Ryegrass (*Lolium perenne* L.) Performance Groups Using Electrolyte Leakage Assay (PRYEFRZELA1)

Objective:

To test the freezing tolerance characteristics, under acclimated and non-acclimated conditions, of 5 genotypes representing high performance perennial ryegrass and 5 genotypes representing low performance perennial ryegrass based on turfgrass performance and quality results obtained from the Maine (Orono) NTEP location.

Experimental material:

- Acclimated and non-acclimated cultivars representing high performance perennial ryegrass:
  - Repell III
  - Prelude III
  - Palmer III
  - Top Hat
  - LRF-94-C8

- Acclimated and non-acclimated cultivars representing low performance perennial ryegrass:
  - Linn
  - DSV NA 9402
  - DSV NA 9401
  - Pennfine
  - SR-4010 (SRX 4010)

- 660, 14 ml polypropylene test tubes (FALCON 17X100 mm) for plant samples.

- 5, 14 ml polypropylene test tubes (FALCON 17X100 mm) for thermocouples.

Acclimated plant material was maintained in containers (5 cm in diam by 17.8 cm deep) in a cool greenhouse (55-60°F) from 19 Oct 1998 to 9 Dec 1998. On 9 December, the material was transferred to the South Deerfield farm and placed

in an unheated (poly) hoop house with the ends opened for air circulation. Plant containers were placed on the floor of the hoop house in blocks by cultivar and all wrapped together with Corning 3.5" house insulation (R13).

Non-acclimated plant material was maintained in containers 5 cm in diam. by 17.8 cm deep in a greenhouse (61-68°F). Containers were placed on a wire shelf, approximately 50 cm off the floor, in blocks by cultivar.

Test temperatures: Non-frozen control (+5°C), -3, -5, -7, -9, -11, -13,-15, -17, -19, and -21°C.

Replications: 3 groups of 5 crowns per replicate container

Protocol:

Pre Day 1 (preparation):

1. Label 660 test tubes for plant material using the following code:

2 environments	A - acclimated N - non-acclimated
10 cultivars	C1-C10
11 temperatures	Non-frozen control (+5°C), -3, -5, -7, -9, -11, -13, -15, -17, -19, -21°C
3 test tubes/cv/env/temp	1, 2, 3

Example: Acclimated, cultivar C1, +5°C, test tube 1.

A	C1	+5°	1

Place test tubes in test tube holders by temperature, grouping acclimated cultivars in one holder and non-acclimated cultivars in another holder. That is, each test tube holder should have 3 test tubes of each acclimated cultivar or 3 test tubes of each non-acclimated cultivar for a total of 30 test tubes in each holder. All test tubes in any one holder should be marked with the same temperature value.

2. Consecutively number 5 test tubes for thermocouples.

3. Fill non-frozen control test tubes, labeled +5, with 5 ml deionized water and place in the refrigerator (+5°C) uncovered. Fill test tubes labeled -3 through -21 and the 5 test tubes marked for thermocouples with 4 ml deionized water and place in the refrigerator uncovered.

4. The day before Day 1 place all test tubes, except non-frozen control, in the freezer at -20°C until frozen, then ramp freezer temperature to -2°C and leave over night. Leave non-frozen control test tubes in the refrigerator.

5. Place 1000 ml of deionized water in a beaker in the refrigerator to cool.

6. Label 20 petri dishes with acclimation (non-acclimation) and cultivar code.

Day 1 (Harvesting of material & Preparation of samples):

Harvesting of material:

1. Harvest 12 containers (includes 1 extra if needed) of each cultivar of acclimated material and 12 containers (includes 1 extra if needed) of each cultivar of non-acclimated material.

2. Starting with the acclimated material (handle one container at a time), wash soil from roots of turf material in cold tap water, wrap in moist toweling, place in properly labeled zip-lock bag (leave unzipped), and refrigerate (+5°C). Repeat using the non-acclimated material.

Preparation of samples:

1. Starting with acclimated material (handle one zip-lock bag at a time), separate material into individual plants, remove roots and stems from the crowns of 15 plants of approximately the same size, and place crowns in the properly labeled petri dish lined with moist filter paper. Loosely cover and keep refrigerated until all crowns have been prepared. Complete preparation of all acclimated material.

2. Once all acclimated crowns are prepared and the frozen test tubes are at -2°C, pull one test tube holder (30 test tubes marked A) at a time from the freezer and place 5 crowns from the properly labeled petri dishes into each test tube. Making sure samples are in direct contact with the ice, add 1 ml cool (from the refrigerator) deionized water to the test tubes and return them to the freezer. Work as quickly as possible to avoid thawing of the ice. Start and complete this step on the same day.

3. From excess crowns, place 5 crowns in each of the 5 test tubes marked for the thermocouples, add 1 ml cool (from the refrigerator) deionized water to each test tube, insert a properly marked thermocouple into each test tube, cover to hold thermocouple in place, and randomly place the test tubes among the samples.

4. DO NOT PLACE NON-FROZEN CONTROL SAMPLES IN TEST TUBES AT THIS TIME. Maintain material in the petri dishes in the refrigerator (+5°C).

Day 2 (continue Preparation of samples):

5. Repeat step 1 using non-acclimated material. Complete preparation of all non-acclimated material.

6. Once all non-acclimated crowns are prepared and the frozen test tubes are at -2°C, pull one test tube holder (30 test tubes marked N) at a time from the freezer and place 5 crowns from the properly labeled petri dishes into each test tube. Making sure samples are in direct contact with the ice, add 1 ml cool deionized water (from the refrigerator) to the test tubes and return them to the freezer. Work as quickly as possible to avoid thawing of the ice. Start and complete this step on the same day.

7. DO NOT PLACE NON-FROZEN CONTROL SAMPLES IN TEST TUBES AT THIS TIME. Maintain material in the petri dishes in the refrigerator (+5°C).

8. Leave frozen samples overnight with freezer set-point at -2°C.

Day 3 (begin freezing schedule):

1. Visually examine tissue samples to ensure freezing has occurred. Lower freezer set-point to the first scheduled test temperature (-3°C). Once tissue temperature has reached -3°C, as measured by the thermocouples, maintain sample temperature for one hour.

2. Remove all -3 samples (2 test tube holders, 30 test tubes each) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.

3. Lower freezer set-point to the next scheduled test temperature (-5°C). Allow tissue temperature to reach -5°C, as measured by the thermocouples, and maintain sample temperature for one hour.

4. Place 5 crowns (non-frozen control samples) from the properly labeled petri dishes in the refrigerator into the control test tubes (60 test tubes) and hold in the refrigerator (+5°C).

5. Remove all -5 samples (2 test tube holders, 30 test tube each) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.

6. Repeat steps 3 and 5 until all scheduled test temperatures up to and including -11°C have been completed. Leave freezer set at -11°C overnight.

Day 4 (continue freezing schedule, begin Electrolyte Leakage Assay):

7. Lower freezer set-point to the next scheduled test temperature (-13°C). Allow tissue temperature to reach -13°C, as measured by the thermocouples, and maintain sample temperature for one hour.

8. Remove all -13 samples (2 test tube holders, 30 test tubes each) from the freezer and place immediately in the refrigerator (+5°C) to thaw overnight.
9. Repeat steps 7 and 8 until the remaining scheduled test temperatures have been completed.

Electrolyte Leakage Assay:

1. Using the non-frozen control samples and the samples thawed overnight from Day 3 freezing (step 6), infiltrate the tissue under partial vacuum for 20 m.

2. Incubate the samples overnight in the refrigerator.

Day 5 (continue Electrolyte Leakage Assay):

3. Shake (approx. 150 rpm) samples incubated overnight (step 2) for 8 h at room temperature.

4. Using the samples thawed overnight from Day 4 freezing (step 9), infiltrate the tissue under partial vacuum for 20 m.

5. Incubate the samples overnight in the refrigerator.

6. Remove samples from the shaker (step 3) and measure the Initial Conductivity (IC) of the leachate in each test tube.

7. Place samples (step 6) in the freezer at -40°C overnight to kill tissue.

Day 6 (continue Electrolyte Leakage Assay):

8. Remove samples from -40°C freezer and let thaw at room temperature (approx. 4 h).

9. Shake (approx. 150 rpm) samples incubated overnight (step 5) for 8 h at room temperature.

10. Shake (approx. 150 rpm) killed samples (step 8) for 2 h at room temperature.

11. Read Final Conductivity (FC) of leachate of killed samples (step 10).

12. Remove samples from the shaker (step 9) and measure the Initial Conductivity (IC) of the leachate in each test tube.

13. Place samples (step 12) in the freezer at -40°C overnight to kill tissue.

Day 7 (finish Electrolyte Leakage Assay):

14. Remove samples from -40°C freezer and let thaw at room temperature (approx. 4 h).

15. Shake (approx. 150 rpm) killed samples (step 14) for 2 h at room temperature.

16. Read Final Conductivity (FC) of leachate of killed samples (step 15).

% Electrolyte leakage EL (%) = (IC/FC) x 100

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