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Development of laboratory-based methods for assessing seedling thermotolerance in pearl millet

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SUMMARY

In this paper, the response to temperature of three physiological processes has been examined in detail in seedlings of sorghum and pearl millet. These have been compared with their field performance under high temperature conditions with the aim of developing laboratory-based screening techniques for seedling thermotolerance. Membrane thermostability, as assessed by electrolyte leakage, seedling re-growth and protein synthetic ability were measured in seedlings exposed to a range of temperatures using a thermal gradient bar. The effect of genotype, seedling age and pre-treatment was examined. Differences between genotypes were only apparent after seedlings had been given a brief high temperature pre-treatment (2 h at 43 °C). Similar results were found with all three methods used and their suitability for use in the screening of large numbers of entries is discussed. Significant correlation was found between the ability of membrane thermostability to acclimate and seedling survival in the field.

Key words: Thermotolerance, electrolyte leakage, growth, protein synthesis, screening techniques.

INTRODUCTION

Temperature is a critical factor for plant growth; extremes of temperatures can cause considerable damage, particularly when they coincide with vulnerable stages of development. The temperature sensitivity of higher plants is not constant throughout their life cycle (Levitt, 1980), with the seed germination and seedling establishment stages being especially susceptible. However, considerable genetic variation exists for the ability both to survive and to grow after exposure to high temperature conditions. The ability to determine the thermosensitivity of a given genotype would provide a means to the genetic improvement of this trait. Screening techniques are required to characterize this variation, to identify appropriate breeding material, and ultimately to select for improved adaptation.

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Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an important cereal grain and fodder crop in many parts of the semi-arid tropics. Poor seedling establishment due to high soil and air temperatures is one of the major causes of seedling death, particularly in the more marginal areas (Peacock *et al.*, 1993). The risk of hot, dry, seedbed conditions during crop establishment is high, with soil surface temperatures often > 50 °C at midday (Gupta, 1986; Howarth, 1991; Peacock *et al.*, 1993). These temperature conditions recur for many days following sowing and cause both thermal injury and the death of young seedlings (O'Neill & Diaby, 1987; Peacock *et al.*, 1993). Injury depends on both the absolute temperature, the duration of exposure and the frequency of exposure to such conditions. A field technique to screen pearl millet entries for their emergence and survival at high soil-surface temperatures has been developed (Peacock *et al.*, 1993). Screening in the target environment is ideal in that relevant climatic variables are potentially available. However there are many difficulties with respect to field screening, particularly in relation to abiotic stress. The natural

environment is not always reliable and is certainly variable both day to day and year to year, screening is often limited to small portions of the year, and the target environment is not always conveniently situated. A laboratory-based technique would have a number of advantages. It could be conducted under controlled conditions, away from the target environment and at any time of year. In addition, the temperature applied to the seedlings can be manipulated depending on the material being assessed. Failure of seedling survival has a number of components and these can not only be examined individually but the correlation between those components established. Dissecting a complex process such as seedling survival of stress into component parts that are under more simple genetic control should facilitate rapid and precise improvements by breeding. The approach used here has employed detailed analysis of the physiological response to temperature of genotypes of contrasting field thermotolerance with the aim of identifying critical processes that can be used for such screening purposes. The challenge is to identify a simple screening test that can be used quickly and easily on large numbers of plants; that is quantitative; that has a heritable response and that displays a close correlation to performance in the field. Such screening techniques can then be used either directly in a breeding programme for improvement of seedling thermotolerance or combined with genetic analysis to identify molecular markers linked to the trait of interest.

A number of physiological processes have been examined in the past for their use in the assessment of tolerance to high temperatures. Cellular membrane dysfunction results from high temperature stress (Levitt, 1980). The leakage of electrolytes into a bathing medium after exposure to stress provides an indication of the cellular membrane damage caused by that stress; the more damage caused by the stress, the more solutes will leak into the bathing medium. Electrolyte leakage has been used to assess stress-induced damage in many species (Sullivan, 1972; Martineau *et al.*, 1979; Blum & Ebercon, 1981; Chen, Shen & Li, 1982; Saadalla, Quick & Shanahan, 1990*a*; Saadalla, Quick & Shanahan, 1990*b*; Tahir & Singh, 1993; Srinivasan *et al.*, 1996). The possibility of using electrolyte leakage to assess seedling thermotolerance in both pearl millet and sorghum seedlings has been investigated here in detail in conjunction with both field screening and also the measurement of seedling growth following exposure to high temperature. In addition, the effect of temperature on protein synthesis has been examined. A thermal-gradient bar capable of treating seedlings at a wide range of temperatures with replication at each temperature point has been used to characterize the response to temperature in detail in four pearl millet entries and two sorghum entries. The effect of seedling age and of acclimation prior to treatment on the gradient bar has also been examined.

MATERIALS AND METHODS

Plant material

Seeds of pearl millet (*Pennisetum glaucum* (L.) R. Br.) and sorghum (*Sorghum bicolor* L. Moench) were supplied by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). The entries used displayed differential field thermotolerance as measured by Peacock *et al.* (1993), and the seed used was of the same batch as that tested in the field. Entry number ICMV 84400 has subsequently been renamed as ICMV 155 and is referred to as ICMV 155 in this paper.

Seeds were surface-sterilized for 5 min in sodium hypochlorite solution (1 % available chlorine), rinsed thoroughly in deionized water and germinated in the dark at 35 °C on vermiculite moistened with deionised water.

For growth and electrolyte leakage studies on 2-d-old seedlings, individual seedlings were carefully placed in thin-walled glass test tubes (12 mm × 75 mm) after 24 h germination, 0·5 cm^3 deionized water was added to each tube and the tubes were returned to 35 °C until required. Studies on older seedlings were conducted using material grown as above but placed in 16 h photoperiod (400 μmol m^{-2} s^{-1}) after 24 h. Immediately before experimentation, intact shoots were dissected and placed in tubes as above.

Plant growth measurements

The effect of temperature on plant growth was determined using a thermal-gradient bar as described in Howarth & Skøt (1994). A temperature gradient from 35 °C to 65 °C was used, seedlings were treated at a given temperature on the thermal gradient bar for 2 h and then returned to a 35 °C incubator in the dark. The change in shoot length (measured from the point of insertion of the mesocotyl with the seed to the tip of the coleoptile) over the subsequent 24 h and 48 h was determined from measurements using a ruler. The effect of various pre-treatments before treatment on the thermal-gradient bar were investigated. Eight seedlings were used at each treatment temperature and each experiment repeated at least three times. Data were analysed using the Maximum Likelihood Programme (MLP; Ross, 1991) and curves fitted using the sigmoid logistic function. LT_{50} values (and standard errors of this fitted coefficient) were determined from the point of inflection of the logistic curve as calculated by MLP. Values obtained for non-pre-treated and pre-treated seedlings were compared using the *t* statistic.

Electrolyte leakage studies

Detailed analysis of the response of seedlings to temperature was conducted using a thermal-gradient bar as described for plant growth studies. Following treatment on the gradient bar, 4·5 ml of deionized water was added to each tube and the tubes incubated for 22 h at 25 °C with shaking. The bathing medium was decanted into a multi-cell seed-analyser tray and the conductivity measured (T1) using an ASA 610 multi-channel conductivity meter. A further 5 ml of deionized water were added to the tubes containing the plant material which were then heated at 99 °C for 0·5 h to kill completely the plant tissue. The final conductivity reading (T2) was taken once the contents of the tubes had returned to 25 °C (2 h at room temperature with shaking). The relative conductivity value for each seedling was calculated as a percentage of the total value as follows:

$$\text{relative conductivity} = \{1-[\text{T1}/(\text{T1}+\text{T2})]\}\times 100,$$

with a value of 100 % representing no injury.

Studies with larger numbers of entries were conducted using a restricted range of temperatures in water baths. Three temperature conditions were used including a control (35 °C) and measurements were taken as above. For this analysis, conductivity values were calculated relative to the non-stressed, control value.

Protein synthesis studies

In vivo labelling of seedlings was carried out using [^{35}S]methionine (44·7 TBq mmol^{-1}; Du Pont) as described by Howarth (1989). Seedlings were incubated at a range of temperatures for 2 h in the presence of 740 kBq of [^{35}S]methionine. Following incubation, labelled seedlings were rinsed in deionized water, blotted dry and homogenized with 4 cm^{-3} g^{-1} (f. wt seedlings) extraction buffer (50 mol m^{-3} Tris, pH 7·5, 6 mol m^{-3} β-mercaptoethanol, 1 mol m^{-3} phenyl methyl sulphonyl fluoride, 1 mol m^{-3} iodoacetate, 0·1 mol m^{-3} EDTA). Sodium dodecyl sulphate was then added to a final concentration of 1 % (v/v). The homogenate was then heated to 100 °C for 5 min and subsequently centrifuged to 12000 ***g*** for 10 min at 4 °C. Aliquots of the supernatant were taken to assess total uptake of radio-label, protein content (Lowry *et al.*, 1951) and radioactivity incorporated into protein (Mans & Novelli, 1960).

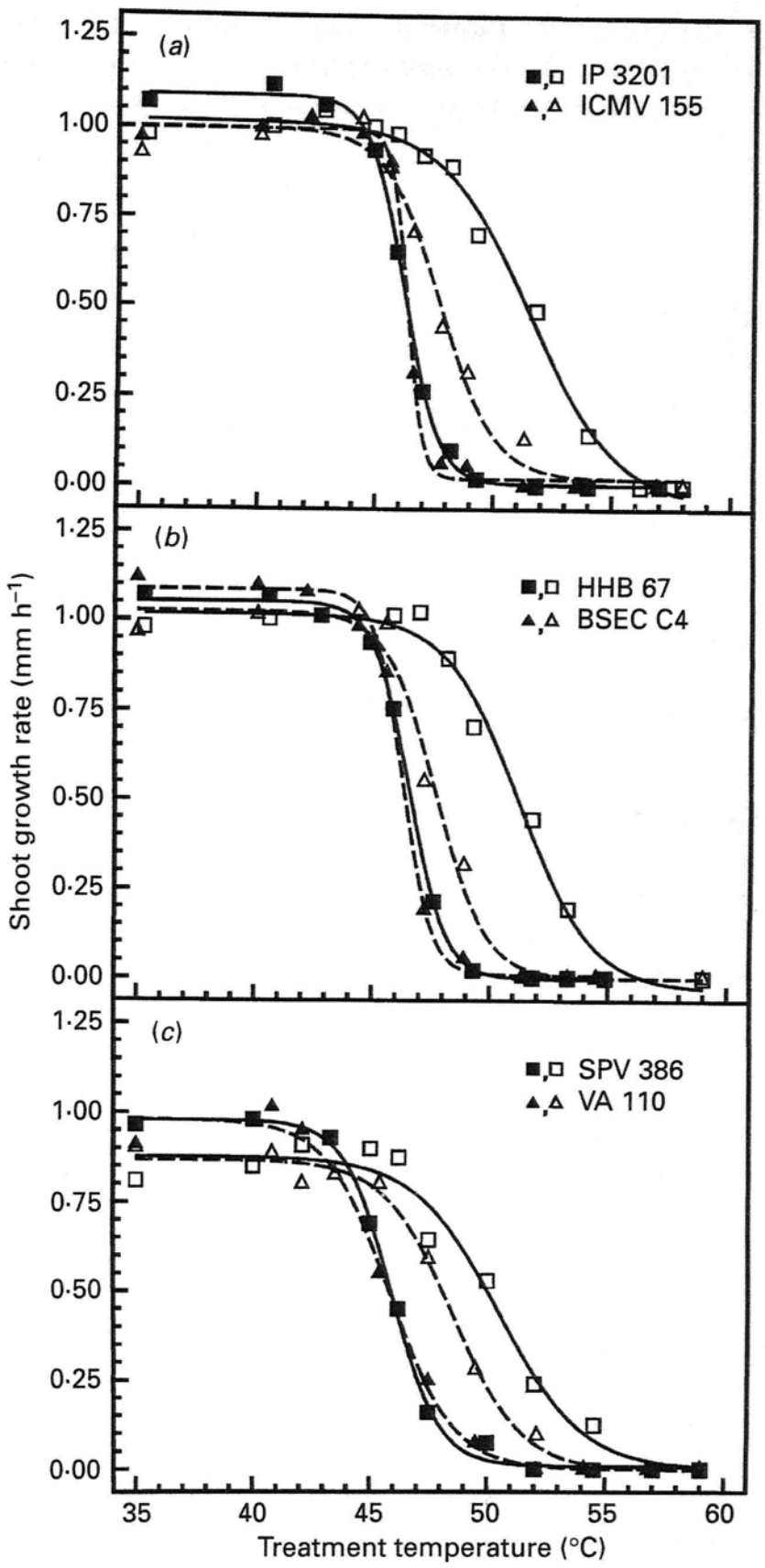

Figure 1. Effect of treatment temperature on the subsequent growth of sorghum and pearl millet seedlings. Two-d-old seedlings were grown individually in test tubes, and eight seedlings were exposed to each of the temperatures indicated using a thermal gradient bar for 2 h either with no pre-treatment (closed symbols) or immediately following a 2 h pre-treatment at 43 °C (open symbols). Seedlings were returned to 35 °C following treatment and the subsequent growth measured after 24 h. The experiment was repeated three times for each entry, the mean shoot growth rate determined (i.e. 24 seedlings per temperature point) and sigmoid logistic curves fitted. Standard errors for each point did not exceed 0·05. (*a*) Pearl millet entries IP 3201 (——, ■, □), ICMV 155 (- - - -, ▲, △). (*b*) Pearl millet entries HHB 67 (——, ■, □), BSEC C4 (- - - -, ▲, △). (*c*) Sorghum entries SPV 386 (——, ■, □), VA 110 (- - - -, ▲, △).

RESULTS

Growth measurements

The response of seedling growth by 2-d-old seedlings of both pearl millet and sorghum following treatment at a range of temperatures on the thermal gradient bar is shown in Figure 1. Seedlings were placed in the thermal gradient bar either with or without a prior heat-shock pre-treatment. After 2 h of treatment at the temperatures indicated, the

Table 1. *LT_{50} values determined from the sigmoid logistic curves fitted to the data obtained for the effect of temperature on growth rate and electrolyte leakage in 2-d-old seedlings of sorghum and pearl millet*

	Growth		Electrolyte leakage	
Entry	Non-acclimated LT_{50} (SE)	Acclimated LT_{50} (SE)	Non-acclimated LT_{50} (SE)	Acclimated LT_{50} (SE)
Sorghum				
VA 110	45·89 (0·22)	48·04 (0·20)	46·60 (0·09)	48·71 (0·11)
SPV 386	45·95 (0·09)	50·01 (0·48)	46·70 (0·11)	50·72 (0·13)
Pearl millet				
BSEC C4	46·30 (0·09)	47·68 (0·20)	47·34 (0·29)	49·08 (0·09)
HHB 67	46·58 (0·05)	51·25 (0·35)	47·43 (0·19)	51·74 (0·14)
IP 3201	46·20 (0·05)	51·52 (0·30)	47·63 (0·23)	52·09 (0·08)
ICMV 155	46·33 (0·05)	47·71 (0·21)	47·16 (0·23)	49·62 (0·15)

Seedlings were either pre-treated for 2 h at 43 °C immediately before temperature treatment (acclimated) or exposed to the treatment temperature directly (non-acclimated).

Table 2. *Effect of acclimation temperature on LT_{50} values determined from sigmoid logistic curves fitted to the data obtained for the effect of temperature on growth rate and electrolyte leakage in 2-d-old seedlings of sorghum and pearl millet*

Entry	Non-acclimated LT_{50} (SE)	40 °C 2 h LT_{50} (SE)	43 °C 2 h LT_{50} (SE)	45 °C 2 h LT_{50} (SE)
Growth				
SPV 386	45·39 (0·14)	46·33 (0·26)n.s.	50·85 (0·53)***	50·51 (0·53)***
Electrolyte leakage				
IP 3201	47·64 (0·10)	47·78 (0·12)n.s.	52·30 (0·22)***	51·78 (0·19)***
ICMV 155	47·15 (0·15)	48·25 (0·35)*	49·34 (0·23)***	49·56 (0·27)***
SPV 386	46·54 (0·14)	47·02 (0·21)n.s.	50·98 (0·26)***	50·50 (0·35)***

Seedlings were either given no pre-treatment (non-acclimated) or pre-treated for 2 h at either 40 :C, 43 °C or 45 °C immediately before treatment at a range of temperatures on the thermal gradient bar. The mean LT_{50} obtained from the non-acclimated seedlings was compared statistically with the mean LT_{50}s obtained from pre-treated seedlings. n.s., non-significant at $P = 0{\cdot}05$; * significant at $P = 0{\cdot}05$; ***significant at $P = 0{\cdot}0001$.

seedlings were returned to 35 °C and their subsequent shoot growth rate determined. When no pre-treatment was given, treatment temperatures > 45 °C were detrimental to further growth in all four peal millet entries, with a very rapid decline in growth rate after exposure to temperatures above this. Treatment temperatures > 50 °C resulted in no further growth of the seedlings. Also shown in Figure 1 is the effect of a pre-treatment period of 2 h at 43 °C immediately before treatment on the thermal gradient bar. Not only was considerable acclimation obtained following such a pre-treatment but it also revealed genotypic variation. For example, seedlings of both pearl millet Ip 3201 and HHB 67 were able to survive temperatures of up to 54 °C after such acclimation whereas seedlings of ICMV 155 and BSEC C4 had considerably reduced growth rates at temperatures > 50 °C. This difference in ability to survive high temperatures agrees with the field thermosensitivity of these entries (Peacock *et al.*, 1993). Sorghum seedlings (entries SPV 386 and VA 110) displayed a greater thermosensitivity with a reduction in growth rate after exposure to temperatures > 40 °C with no pre-treatment. Again, acclimation to high temperature was apparent in seedlings after a pre-treatment; pre-treated seedlings displayed no reduction in growth rate after treatment at temperatures of up to 47 °C as compared with pre-treated seedlings exposed to lower treatment temperatures. Interestingly, although the pre-treatment resulted in considerable acclimation, growth rates at lower temperatures (< 45 °C) were lower than in non-pre-treated seedlings.

Sigmoidal logistic curves fitted the response of growth as a function of treatment temperature. The most critical region of the response and the biggest difference between entries occurred at temperatures which resulted in 50% injury. This corresponds with the point of inflection of the logistic curve. From this it is possible to determine LT_{50} values, and these can be used to characterize entries (Table 1). LT_{50} values obtained for pearl millet entries with no acclimation were not significantly different ($P = 0{\cdot}05$). However, LT_{50} values obtained from acclimated seedlings were significantly different from those obtained from non-acclimated seedlings ($P =$

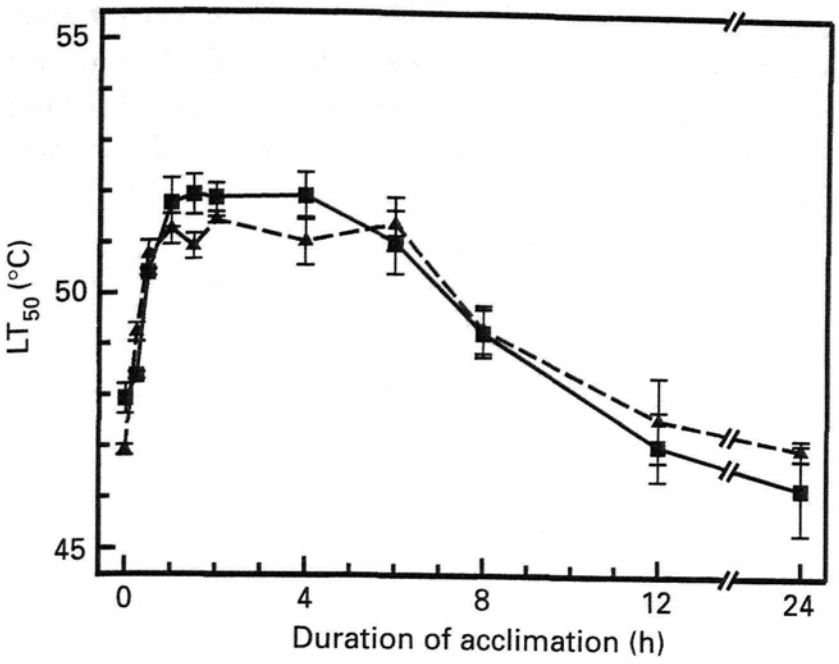

Figure 2. The effect of the duration of acclimation at 43 °C on the LT_{50} obtained for growth (——, ■) and electrolyte leakage (----, ▲) with pearl millet entry IP 3201. Two-d-old seedlings were treated at a range of temperatures as described in the 'Materials and Methods' with either no pre-treatment or a pre-treatment at 43 °C for a range of timings between 30 min and 24 h. Logistic sigmoid curves were fitted to the data obtained from each time point and the LT_{50} determined. Error bars represent 1 SE of the mean.

0·001). Clear differences between entries in this ability to acclimate are evident; following acclimation, the LT_{50} values for HHB 67 and IP 3201 increased by at least 4·5 °C whereas those for BSEC C4 and ICMV 155 increased by < 1·4 °C. Sorghum seedlings with no acclimation had significantly lower LT_{50} values compared to pearl millet but again, showed a significant increase in LT_{50} after acclimation.

In the above experiments, a 2 h 43 °C pre-treatment was used immediately before placing seedlings on the thermal gradient bar. The effect of the pre-treatment temperature on the thermosensensitivity of seeding growth was examined with sorghum seedlings. A pre-treatment at 40 °C for 2 h before the gradient-bar treatment resulted in little induced thermotolerance (Table 2). Either 43 °C or 45 °C for 2 h induced maximal thermotolerance. The effect of the duration of the pre-treatment period on acclimation is shown in Figure 2 for pearl millet entry IP 3201. Acclimated thermotolerance was induced rapidly, was maximal by 1 h at 43 °C and remained stable for up to 6 h. Acclimation periods > 6 h resulted in reduced thermotolerance and by 12 h at 43 °C thermotolerance was similar to the non-acclimated control (Fig. 2). Recovery periods at 35 °C between pre-treatment and treatment on the thermal gradient bar result in a loss of induced thermotolerance (Howarth & Skøt, 1994). The duration of treatment on the thermal gradient bar also affected the response of seedlings to temperature. A 2 h treatment period was chosen, either with or without a 2 h acclimation at 43 °C, was used as this both gave a reasonable response and was similar to the duration of high temperatures at midday in the field (Howarth, 1991). Longer acclimation periods at lower temperatures were not examined.

Electrolyte leakage

Electrolyte leakage was used as an independent measure of the effect of temperature on pearl millet and sorghum seedlings. As for growth studies, seedlings were treated on a thermal gradient bar for 2 h either with or without a prior heat-shock pre-treatment. The seedlings were then incubated in deionized water, and the damage caused by temperature assessed by the measurement of the conductivity of the bathing medium relative to the total conductivity value of the bathing medium obtained when the seedlings were killed. The leakage of solutes from the cell contents to the bathing medium provides an indirect measure of membrane damage. In Figure 3, relative conductivity is plotted against treatment temperature for the four pearl millet and the two sorghum entries. Intact, 2-d-old dark-grown seedlings were used for these analyses. A value of between 82·5% and 88·2% was obtained for seedlings maintained at 35 °C. With no pre-treatment, a change in the relative conductivity was found after exposure to treatment temperatures > 45 °C and treatment temperatures > 50 °C resulted in maximal electrolyte leakage (Fig. 3). Again, a sigmoidal logistic curve fitted the data well and LT_{50} values were calculated (Table 1). Little difference was found in the values obtained for non-acclimated material but clear genotypic differences were found in the ability to acclimate. A pre-treatment at 40 °C for 2 h before the gradient-bar treatment resulted in a slight induction of thermotolerance (Table 2) but either 43 °C or 45 °C for 2 h induced maximal thermotolerance in all three entries examined. Interestingly, the most thermosensitive entry (ICMV 155) was able to acclimate to a significant degree ($P = 0{\cdot}05$) after a 40 °C pre-treatment whereas the other two entries required a higher temperature of pre-treatment to obtain a significant difference from the non-acclimated control. The effect of duration of acclimation at 43 °C on LT_{50} values for electrolyte leakage are shown in Figure 2. Acclimated thermotolerance was induced by 1 h at 43 °C and started to decline after 4 h of pre-treatment. After 12 h at 43 °C, thermotolerance was similar to the non-acclimated control (Fig. 2). The greatest difference between entries was found with seedlings that had been acclimated for 2 h at 43 °C immediately before treatment on the thermal-gradient bar for 2 h (Fig. 3, Table 1). The pearl millet entries IP 3201 and HHB 67 displayed a superior ability to acclimate to high temperature, as did the sorghum SPV 386. These were the same entries that showed greater ability to acclimate to high tem-

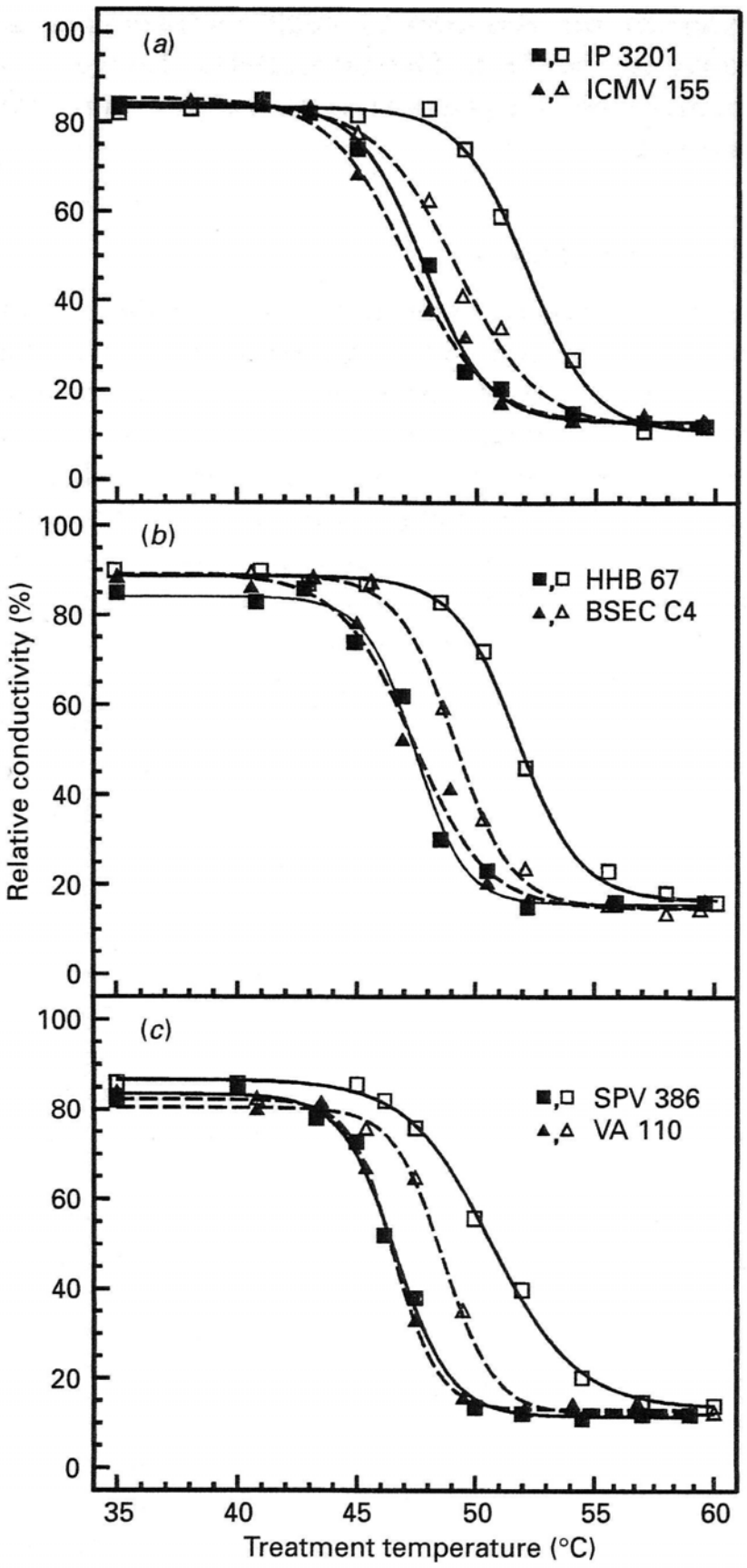

Figure 3. Effect of treatment temperature on electrolyte leakage from sorghum and pearl millet seedlings. Two-d-old seedlings were grown and treated as in Figure 1 for 2 h either with no pre-treatment (closed symbols) or immediately following a 2 h pre-treatment at 43 °C (open symbols). Electrolyte leakage was measured as described in 'Materials and Methods'. The experiment was repeated three times for each entry, the mean relative conductivity determined for each temperature point and sigmoid logistic curves fitted. (*a*) Pearl millet entries IP 3201 (——, ■, □), ICMV 155 (----, ▲, △). (*b*) Pearl millet entries HHB 67 (——, ■, □), BSEC C4 (----, ▲, △). (*c*) Sorghum entries SPV 386 (——, ■, □), VA 110 (----, ▲, △).

perature when the response of growth was assessed (Fig. 1, Table 1). The LT_{50} values obtained using conductivity measurements were always higher, both with and without acclimation, than those obtained from growth studies but the trends obtained were similar.

Older seedlings were also examined for electrolyte leakage after treatment on the thermal gradient bar.

Table 3. *LT_{50} values calculated from electrolyte leakage measurements of shoots from older seedlings exposed to a range of temperatures on the thermal gradient bar either without (non-acclimated) or with (acclimated) a 2 h pre-treatment at 43 °C*

Entry	Non-acclimated LT_{50} (SE)	Acclimated LT_{50} (SE)
Sorghum		
VA 110	50·83 (0·28)	52·88 (0·15)***
SPV 386	50·58 (0·26)	54·18 (0·15)***
Pearl millet		
BSEC C4	49·01 (0·19)	50·03 (0·25)*
HHB 67	50·60 (0·21)	53·85 (0·26)***
IP 3201	50·41 (0·16)	54·20 (0·17)***
ICMV 155	49·10 (0·21)	50·75 (0·18)*

Sorghum seedlings used were 7-d-old and pearl millet 5-d-old. Superscripts as for Table 2.

Seedlings at the two-leaf stage were used (5 d after sowing for pearl millet and 7 d after sowing for sorghum) and intact shoots were carefully dissected at the point of insertion of the shoot with the seed, keeping the shoot base under water before placing in the treatment tube. Sigmoidal logistic curves fitted the response of conductivity against treatment temperature (data not shown) and LT_{50} values were calculated (Table 3). With no pre-treatment, older seedlings were more thermotolerant than 2-d-old seedlings although more variation in the LT_{50} values was obtained (Table 3). An increase in LT_{50} of at least 3 °C was found between 5-d-old non-acclimated pearl-millet seedlings and 2-d-old seedlings. This increase in LT_{50} for non-acclimated seedlings was even greater in the sorghum entries examined. Older seedlings, however, not only displayed the ability to acclimate but also differences between entries were found after acclimation. Again those entries displaying greater thermotolerance in the field (IP 3201, ICMV 155 and SPV 386) exhibited the greatest increase in LT_{50} after a 2 h pre-treatment at 43 °C.

For screening, determination of LT_{50} values is relatively time consuming for the large numbers of entries involved. The curves obtained from the thermal-gradient-bar experiments were examined to determine what single temperatures to use with larger numbers of seedlings. It was decided to use 48 °C for 2 h as the determining temperature for 2-d-old seedlings, either with or without acclimation at 43 °C for 2 h. For older seedlings (5-d-old), 52 °C (without or without acclimation at 43 °C for 2 h) was used as the determining temperature. In addition, a control set of seedlings was treated at 35 °C so that the relative conductivity could be calculated. Twenty pearl-millet entries for which detailed field analysis has already been undertaken (Peacock *et al.*, 1993) were examined under these conditions (Figure 4). A correlation coefficient (r^2) of 0·78 was obtained

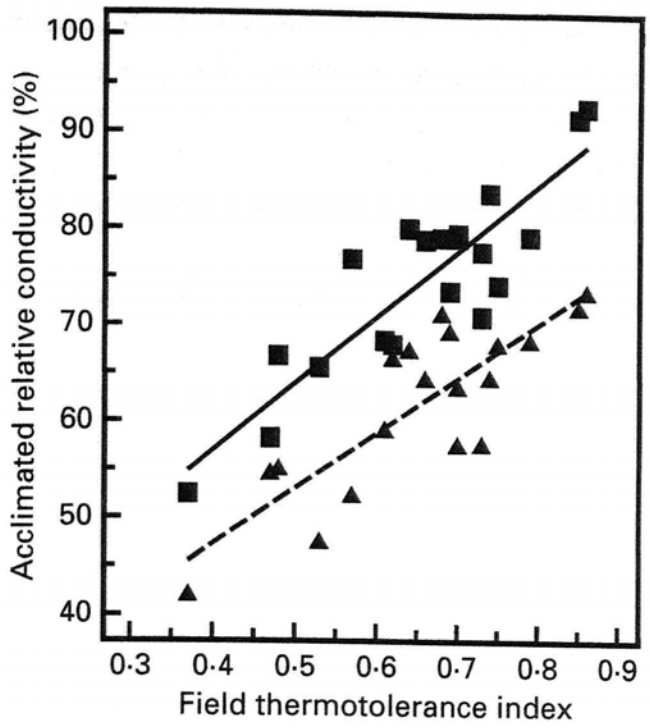

Figure 4. Comparison of the field thermotolerance index obtained for 19 entries of pearl millet (from Peacock *et al.*, 1993) with the relative conductivity value obtained from seedlings of the same entries exposed to either 2 h at 43 °C followed by 2 h at 48 °C (2-d-old seedlings, ----, ▲) or 2 h at 43 °C followed by 2 h at 52 °C (5-d-old seedlings, ——, ■).

between the relative conductivity at 48 °C with acclimation for 2-d-old seedlings and the field thermotolerance index previously calculated. Five-d-old seedlings gave a correlation coefficient of 0·68 between acclimated relative conductivity and field thermotolerance. The correlation coefficient obtained between the results from 2-d- and 5-d-old seedlings was 0·58. This indicates that the stage of development affects the thermotolerance of an entry. However there were differences in the methodologies used for the 2-d-old and 5-d-old seedlings; only the shoots of the 5-d-old seedlings were used whereas at 2-d-old, intact seedlings were treated.

Non-acclimated relative conductivity values gave correlation coefficients $< 0{\cdot}1$ with field thermotolerance values for both 2-d-old and 5-d-old seedlings. It would appear that it is the ability to acclimate that is of critical importance in the survival of high temperature.

Protein synthesis

The effect of temperature on protein synthesis was assessed by incubating seedlings with [^{35}S]methionine and measuring its incorporation into protein during the temperature treatment. The amount of protein synthesis detected was normalised with respect to the total protein content measured for that sample. The uptake of radioactivity was also measured and no significant difference was found between samples indicating that the reduction in incorporation of radioactivity into protein was not caused by a reduced amount of radioactivity in those samples. Treatment temperatures of up to 45 °C resulted in a slight increase in protein synthesis as

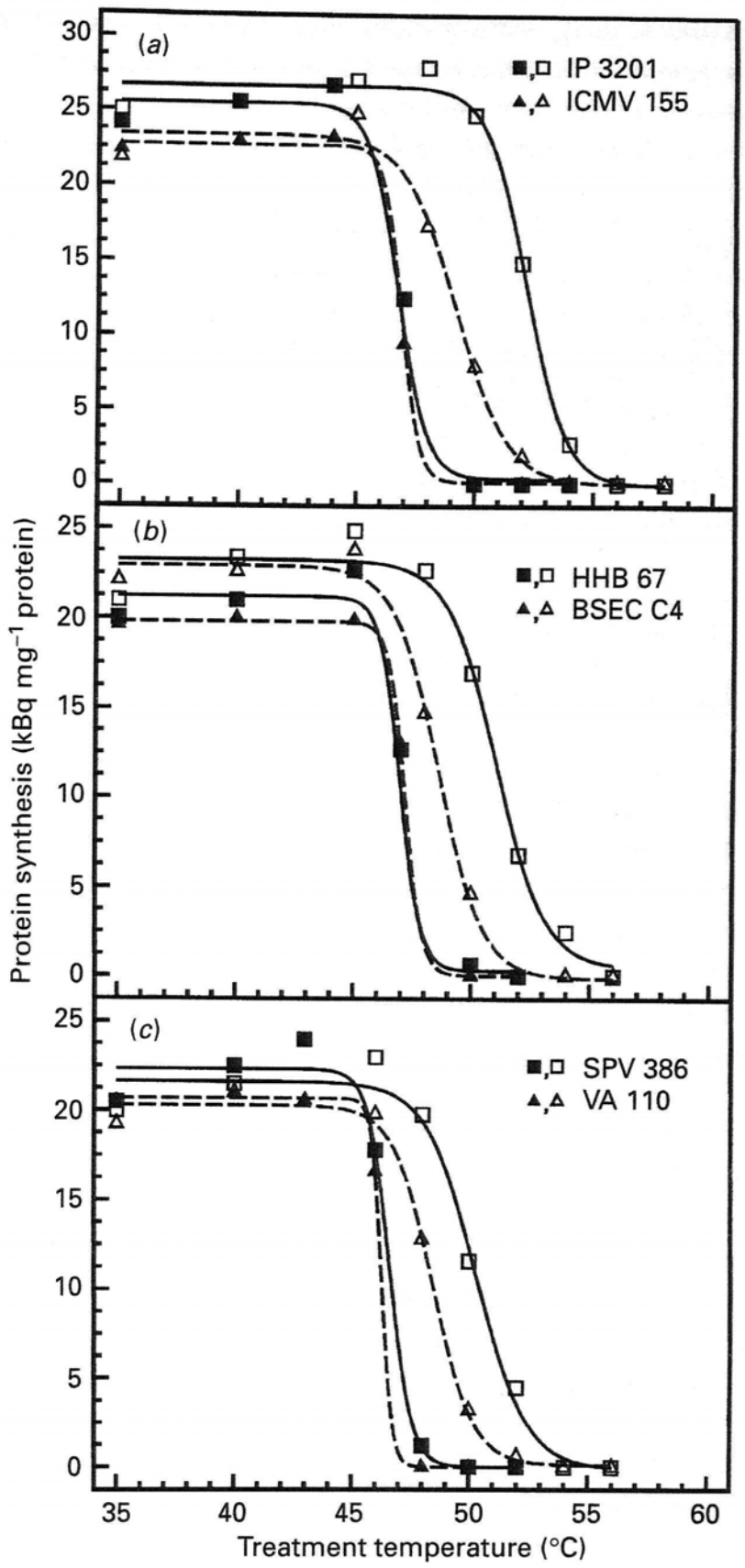

Figure 5. Effect of treatment temperature on protein synthesis by sorghum and pearl millet seedlings. Two-d-old seedlings were treated for 2 h at a range of temperatures either with no pre-treatment (closed symbols) or immediately following a 2 h pre-treatment at 43 °C (open symbols). [^{35}S] methionine was included in the incubation medium in the final 2 h and seedlings were homogenized immediately, following treatment. Protein synthesis determined as described in 'Materials and Methods' and expressed per mg total protein in the sample. The experiment was repeated three times for each entry, the mean protein synthesis determined for each temperature point and sigmoid logistic curves fitted. (*a*) Pearl millet entries IP 3201 (——, ■, □), ICMV 155 (----, ▲, △). (*b*) Pearl millet entries HHB 67 (——, ■, □), BSEC C4 (----, ▲, △). (*c*) Sorghum entries SPV 386 (——, ■, □), VA 110 (----, ▲, △).

compared to that found at the standard growth temperature of 35 °C but treatment temperatures above this resulted in a rapid cessation of protein synthesis with barely detectable synthesis at 48 °C (Figure 5). Logistic sigmoid curves were fitted to the

Table 4. *LT_{50} values calculated from the sigmoid logistic curves fitted to the data obtained for the effect of temperature on protein synthesis in 2-d-old and 5-d-old seedlings of sorghum and pearl millet*

	2-d-old seedlings		5-d-old seedlings	
Entry	Non-acclimated LT_{50} (SE)	Acclimated LT_{50} (SE)	Non-acclimated LT_{50} (SE)	Acclimated LT_{50} (SE)
Sorghum				
VA 110	45·42 (0·19)	48·03 (0·41)***	n.d.	n.d.
SPV 386	46·64 (0·29)	50·31 (0·30)***	n.d.	n.d.
Pearl Millet				
BSEC C4	47·20 (0·48)	48·60 (0·15)*	49·10 (0·41)	51·44 (0·50)*
HHB 67	46·98 (0·14)	51·11 (0·29)***	49·60 (0·32)	53·56 (0·42)***
IP 3201	46·77 (0·30)	52·24 (0·19)***	50·61 (0·35)	54·03 (0·38)***
ICMV 155	46·85 (0·26)	49·24 (0·21)***	50·61 (0·23)	51·94 (0·32)*

Seedlings were either pre-treated for 2 h at 43 °C immediately before temperature treatment (acclimated) or exposed to the treatment temperature directly (non-acclimated). [^{35}S] Methionine was included in the final 2 h of treatment and its incorporation into protein determined. Superscripts as for Table 2.

data obtained and LT_{50} values calculated (Table 4). For treatment temperatures below 45 °C, the logistic curve did not fit the data as well as it did for both the growth and conductivity studies, and the standard errors obtained for the LT_{50} values were in general higher than those obtained from either of the other techniques. The effect of a pre-treatment of 2 h at 43 °C immediately before the addition of the [^{35}S] methionine is also shown. This resulted not only in significant acclimation in all entries but also in genotypic differences. The entries IP 3201, HHB 67 and SPV 386 all exhibited an enhanced ability to acclimate the process of protein synthesis to high temperature. The entry BSEC C4 showed the least ability to acclimate. Five-d-old pearl-millet seedlings were also examined for their ability to synthesize protein at a range of temperatures, both with and without pre-treatment and the LT_{50} values obtained are shown in Table 4. Not only was protein synthesis by older seedlings more thermotolerant with no pre-treatment but protein synthesis also acclimated to high temperature with entries HHB 67 and IP 3201 displaying again the greatest ability to acclimate.

DISCUSSION

The results above indicate that the response of electrolyte leakage to temperature not only correlates well with the effect of temperature on seedling growth and with the ability to synthesize protein at high temperature but also with field thermotolerance. This indicates that any of these laboratory techniques could be used for the large scale screening of the thermal sensitivity of seedlings of both sorghum and pearl millet. The practicalities and limitations of conducting each method as a screening technique are considered below. The use of electrolyte leakage for assessing stress tolerance is not new; a method for measuring temperature tolerance in sorghum leaf discs using conductivity measurements was described previously by Sullivan (1972). This was modified here to measure the electrolyte leakage of whole seedlings or intact shoots rather than using leaf discs. This has the advantage in that cut surfaces are minimized and seedlings are only damaged by the temperature treatment. This methodology was also more appropriate for the study of the effects of temperature on seedlings. Electrolyte leakage is not only dependent on the temperature of the treatment given and its duration but also on the species and cultivar used, its stage of development, and on the parameters of the measurement itself, for example the tissue type, length of time in and temperature of the bathing medium. In addition, as shown here, pre-treatment of the tissue also affected its response to temperature.

Plants show considerable capacity to acclimate to cope with changes in the environment, whether they be seasonal or diurnal variations. At least two types of high-temperature acclimation processes are apparent: Firstly, long term heat-hardening induced on a time scale of days or weeks at moderately high temperatures and secondly, short term thermotolerance induced by exposure for a few hours or less to high temperature. The latter is thought to be involved with coping with diurnal changes in temperature and results in tolerance of extreme high temperatures. The mechanisms controlling these two acclimation processes are likely to be very different. Most studies using electrolyte leakage have examined the effect of long-term heat-hardening. Chen *et al.* (1982) expressed heat tolerance in genotypes of bean, soybean, potato and tomato on the basis of electrolyte leakage from leaf discs either after a given duration at a range of temperatures or after a range of exposure times to a given temperature; they found the greatest difference between genotypes after an acclimation period of 24 h and 35 °C. In the work presented here, a far shorter pre-treatment at a relatively higher tem-

perature also resulted in induced heat tolerance and indeed a longer pre-treatment at such a temperature was detrimental to both seedling growth and membrane thermostability. The material to be tested must have been exposed to the same thermal history if the results are to be comparable, particularly if leaf material harvested from the field is used. Electrolyte leakage is also affected by the water status and the drought tolerance of a genotype (Blum & Ebercon, 1981). These factors must be considered in the experimental design; they make comparison of the results obtained between different studies difficult. Most studies examine the effect of a single temperature and selection of an appropriate temperature to use as a test temperature requires defined characterization as shown here. Detailed studies using a thermal-gradient bar not only provided a precise LT_{50} value for each entry tested, but enabled characterization of the response to temperature thus enabling the selection of the optimum temperature for making single, critical measurements as required for a screening technique. Once this has been done however, extensive measurements can be taken quickly and inexpensively in a controlled and accurate manner. Close correlation was found here between field thermotolerance and electrolyte leakage from acclimated seedlings treated at 48 °C (2-d-old) or 52 °C (5-d-old). A population cross between two contrasting pearl-millet entries identified here (IP 3201 and ICMV 155) has now been made and the progeny from this are currently being examined both for seedling thermotolerance in the field and for electrolyte leakage after exposure to high temperature to further validate the results found here. A useful screening technique needs to be able to differentiate entries with relatively small differences in performance. The conditions used to assess heat tolerance also need to be representative of the field conditions likely to be encountered. For example, in this study both the duration and temperature of acclimation and treatments used were similar to those likely to be encountered by seedlings in the field.

Comparisons between heat tolerance as measured by electrolyte leakage and final grain yield in the field under high temperature conditions have produced conflicting results. Sullivan & Ross (1979) found a good correlation between yield in a hot and dry environment and membrane thermostability. However, no significant correlation was found in similar studies using either soybean (Martineau *et al.*, 1978) or spring wheat (Shanahan *et al.*, 1990). In these studies, field grown leaves were used and tested at a single temperature with no acclimation pre-treatment. Such material is also likely to be affected by variation in environmental conditions and as shown here, the results obtained are highly dependent on the age of the tissue and whether the tissue has been exposed to high temperature before sampling. In both these studies however, material designated as heat tolerant as determined by electrolyte leakage in general yielded more at sites where heat stress during grain fill was prevalent then material with poor membrane thermostability. In winter wheat (Saadala *et al.*, 1990*b*), electrolyte leakage after exposure to 49 °C for 1 h was assessed in seedlings grown under controlled conditions and hardened for 48 h at 34 °C. Although this provided excellent correlation with membrane thermostability in plants sampled at anthesis (Saadala *et al.*, 1990*a*) and plants with high membrane thermostability had increased grain yield, no consistent relationship was found in the field. It is possible that the plants in the field were not exposed to sufficiently high temperatures for any differences to be realised. Membrane thermostability is particularly significant only at those extreme temperatures where membrane disruption is likely. For the purposes of this paper, where screening techniques for the ability of pearl millet seedlings to survive high temperatures rather than the effect of temperature on yield is being considered, a far better correlation is found between membrane thermostability and field performance. The reduction in grain yield due to high temperature involves many different aspects of physiology, for example photosynthetic rate, assimilate partitioning and growth rate. Only at extreme temperatures will membrane thermostability affect these processes.

Close correspondence in the results obtained from electrolyte leakage screening was found with those from the re-growth test although the LT_{50} values obtained were always higher when considering electrolyte leakage in both non acclimated and acclimated material. Growth after a temperature treatment integrates both the response to stress and the ability to recover from it, whereas electrolyte leakage is only looking at one component of damage during the stress conditions itself. Electrolyte leakage also measures the damage to all the cells in the seedling whereas only a small region of cells at the shoot basal meristem is involved in shoot growth. The lower LT_{50} values for re-growth following temperature treatment indicates that shoot growth is more sensitive to the effects of temperature than membrane thermostability and that not all cells are damaged equally during stress. The regrowth test has one advantage in that it is non-destructive and surviving seedlings can be planted out after treatment. The method has only been tested here for 2-d-old seedlings; further work would be needed to test its suitability for older material. The measurements of protein synthesis provide another assessment of the effect of temperature on seedlings. Protein degradation, and the consequent effects on both enzyme activity and membrane function, results from exposure to high temperature. Protein synthesis is a very thermosensitive metabolic process and thus the ability to synthesize protein at high

temperature is critical in the survival process. A very rapid decrease in the ability to synthesize protein was found above 45 °C and although it was possible to characterize entries on the basis of their LT_{50}, the selection of a single temperature for assessing a large number of entries would not be straightforward owing to the sharp gradient of the logistic curves obtained. This method also employs the use of radioactivity in its determination which limits its utility as a screening technique. A laboratory screening technique measuring the thermosensitivity of sorghum germination based on embryo protein synthesis has been reported previously (Ougham & Stoddart, 1985) and a strong correlation was found between the ability of imbibing embryos to synthesize protein at temperatures > 40 °C and germination at high temperatures.

Little difference was found between non-acclimated entries using any of the methods, indicating that the extent of heat tolerance for plants grown at control temperatures is essentially the same. The ability to acclimate rapidly to the effects of a change in temperature is much more critical in determining whether a given entry will survive or not. Although the mechanism of acclimation to high temperature is not fully understood, it is not thought to be caused by temperature-induced changes in lipid content or fatty acid composition of the membranes. An alternative possibility is that the marked changes in protein synthesis that occur during acclimation are involved in the development of thermotolerance. As can be seen here, not only does temperature affect the ability of seedlings to synthesize protein but protein synthesis itself is also capable of acclimating after a heat shock pre-treatment. The metabolic changes that occur during that acclimation period are the key rather than the intrinsic thermotolerance of non-acclimated seedlings. This includes the synthesis of heat shock proteins which have been implicated in the development of thermotolerance in many organisms (Howarth & Ougham, 1993; Vierling, 1991). Both qualitative and quantitative variation in heat-shock protein synthesis has been found in maize (Frova & Gorla, 1993; Jorgensen & Nguyen, 1995) and in sorghum (Howarth, 1989). The potential for the use of such proteins in the development of screening techniques for thermotolerance in pearl millet is currently being investigated.

This study confirmed that genetic variability exists within pearl millet for the ability to survive high temperatures as previously revealed by field screening, and has identified appropriate parameters to use for large scale screening. An understanding of the physiology of seedling response to stress is required to enable the development of such screening techniques. Correlation of a screening technique not only with other critical physiological processes but also with field performance emphasizes its relevance. It is also possible now by using the approaches of genetic mapping and quantitative trait loci (QTL) analysis to screen for thermotolerance by a number of different methods and determine their genetic correlation as well. Molecular marker maps exist for many crops including pearl millet (Liu *et al.*, 1994) and have been used to identify QTLs associated with many traits. A similar approach is currently being used to detect QTLs associated with seedling thermotolerance (Howarth *et al.*, 1994). Genetic mapping the potential physiological and biochemical components of a trait not only provides information on their involvement in that trait but is also a new way of elucidating the mechanism of plant response to temperature. Active collaboration between geneticists, molecular biologists, physiologists, breeders and other relevant disciplines are required to ensure success.

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