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Comparison of cell membrane thermostability and chlorophyll fluorescence parameters for the determination of heat tolerance in ten cabbage lines

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SUMMARY
Cell membrane thermostability (CMT) and chlorophyll fluorescence (CF) were determined for ten lines of cabbage in order to select heat-tolerant varieties, to provide an improved method to screen for heat tolerance, and to determine whether there is a relationship between CMT and CF. Chlorophyll fluorescence parameters, especially variable fluorescence (*Fv*) and the ratio between *Fv* and maximum fluorescence (*Fm*), were found to be better than CMT for screening cabbage lines for heat tolerance. *Fv* values and the *Fv/Fm* ratio of stressed plants corresponded to high heat damage for the varieties 'HRI 002605', 'HRI 003202', and 'HRI 007827'; and to low heat damage for the varieties 'HRI 013011', 'HRI 005237', and 'HRI 006556'. The latter group may therefore be more tolerant of high temperature stress in the tropics. There were significant relationships ($P < 0.05$) between relative injury (RI), an index of CMT, and two of the CF parameters [miniumum florescence (*Fo)* and *Fv/Fm*] under stress conditions (35° – 40°C). This suggests that parameters measured under stress temperatures are more reliable than those measured under non-stressed conditions when determining heat tolerance.

Cabbage (*Brassica oleraceae* L. var. *capitata*.) is a cool-season crop and grows well at 20° – 25°C (Messiaen, 1992; Ghosh and Madhavi, 1998). Normally, for seed production, temperatures between 0° – 10°C are needed for 6 – 8 weeks to induce flowering. Cabbage is one of the few exotic crops for which production and use are increasing rapidly in many tropical countries, especially in West Africa. Techniques are required for the rapid identification of heat-tolerant varieties for commercial production, potential seed production, and breeding programmes in tropical areas. Two tests that have gained in popularity for rapid screening of agricultural crops for high temperature tolerance are cell membrane thermostability (CMT) and chlorophyll fluorescence (CF; Martineau *et al*., 1979; Chauhan and Senboku, 1996; Sipos and Prange, 1986; Maxwell and Johnson, 2000).

The CMT test is based on the fact that the injury inflicted on leaf tissues under high temperatures weakens the cell membrane, which leads to a leakage of electrolytes out of the cell. If the leaf tissue is then washed in de-ionised water, the amount of electrolyte leakage can be determined using a conductivity meter, and higher conductivity values mean more cell membrane injury (Martineau *et al*., 1979). The effectiveness of CMT to screen genotypes for heat tolerance has been reported for some plants (Chen *et al*., 1982; Yeh and Hsu, 2004; Martineau *et al*., 1979; Chauhan and Senboku, 1996).

In addition to the cell membrane, it has been reported that the thylakoid membrane is one of the first components of the photosynthetic apparatus to be damaged by heat (Santarius, 1974; cited in Sipos and Prange, 1986) which normally leads to changes in the level of chlorophyll fluorescence (CF). The ability of thylakoid membranes, which contain the carriers for electron transport, and photosystems I (PSI) and II (PSII), to resist heat damage varies between species and varieties (Sipos and Prange, 1986). Sipos and Prange (1986) further indicated that, by measuring CF, which is the re-emitted energy absorbed by PSII but not transferred to PSI, the efficiency of electron transport in PSII can be determined, which in turn gives an indirect measure of heat tolerance. Thermal damage within PSII is characterised by a marked increase in initial fluorescence (*Fo*) and a decrease in variable fluorescence (*Fv*) and in the ratio of *Fv* to maximum fluorescence (*Fm*; Maxwell and Johnson, 2000; Hansatech, 1996; Havaux, 1995; Sipos and Prange, 1986). These fluorescence parameters have been used to screen plants for tolerance to high temperature (Martineau *et al*., 1979). However, it is not known whether these methods would be effective for screening cabbage for heat tolerance, or whether there is a relationship between the two methods.

The aim of this study was to select heat-tolerant cabbage varieties for growth in the tropics using CMT and CF, and to determine the best method or parameter for future screening of temperature tolerance in cabbage. One further aim was to determine whether there was a relationship between CMT and CF in cabbage.

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MATERIALS AND METHODS

Seeds of ten cabbage lines (accession numbers: 'HRI 011446, 'HRI 013011', 'HRI 002605', 'HRI 003202', 'HRI 003720', 'HRI 005237', 'HRI 006556,' HRI 009617', 'HRI 009837', and 'HRI 007827') obtained from Warwick HRI, University of Warwick, Wellesbourne, UK, were used for this study. The heads of cabbage plants, grown in the previous season, were removed to allow axillary buds to develop on the remaining stalk. One month later, five developed buds were cut from each of the ten lines and rooted in a 1:1 (v/v) perlite-sphagnum moss mix for 3 weeks, after which they were transferred into Levington F2S compost (Scotts Professional, Ipswich, UK) in 4 cm-square modules in modular plastic trays and kept in a closed propagator in a controlled environment room at 20°C with a 12 h photoperiod at 400 μmoles m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After 3 weeks, plants were transferred into 15 cm top-diameter plastic pots, each containing 0.85 kg Levington M2 soil mix (Scotts Professional) and maintained in a glasshouse at 20° – 30°C. Seven weeks after rooting, CMT and CF measurements were made as described below.

Cell membrane thermostability (CMT) test

The procedure used was similar to that described by Chauhan and Senboku (1996) and by Martineau *et al.* (1979). Leaf discs from plants raised at 20º – 30ºC were treated by heating to 50ºC for 15 min and their electrolyte leakage was compared to that of control leaf discs which did not receive the heat treatment The test was repeated on four plants from each of the ten cabbage lines. Two young leaves (the uppermost fully-expanded leaves) from each plant, each approx. 100 cm^2 in area, were used for each assay. The leaves were first washed thoroughly in tap water, then with distilled water to remove any soil particles. Paired adjacent sets (control and heat-treatment) of ten leaf discs were cut from each leaf sample, avoiding the midrib, using a 1 cm-diameter cork-borer and this was repeated for the other leaf. The ten control and ten heat-treated leaf discs were then placed into two separate test-tubes, and washed thoroughly with at least four changes of distilled water. This procedure removed exogenous electrolytes adhering to leaf tissue surfaces and removed endogenous electrolytes released from cut cells at the periphery of the discs. After the final wash, the tubes were drained of excess water. Sufficient water remained on the discs and tube interior to maintain a high humidity. The heat-treatment tubes were then covered with Saran plastic wrap and incubated in a thermostatically-controlled water bath for 15 min at 50°C, while the control tubes were maintained at 25°C for 15 min. After the elevated temperature treatment, the heat-treatment tubes were quickly cooled to 25°C, and both the control and treatment tubes were filled with 15 ml distilled water and incubated overnight for 18 h at 10°C to allow diffusion of electrolytes from the leaf discs. The tubes were then transferred to a water bath at 25°C, the contents mixed thoroughly for 5 s on a vortex mixer, and an initial conductance measurement was made using a conductivity meter (Model 4071; Jenway, Dunmow, UK). After this, both the control and heat-treatment tubes were covered with Saran plastic wrap and autoclaved at 121°C for 15 min to lyse all cells and release all the electrolytes. All the tubes were then cooled to 25ºC, the contents mixed thoroughly, and final conductance measurements were taken. The relative injury (RI) induced as a result of the initial 50°C temperature treatment, was then calculated as follows:

$$RI\ (\%) = \{1 - ([1 - (S_1/S_2)] / [1 - (C_1/C_2)]\} \times 100$$

where, S and C refer to conductance values for the heat-treatment and control tubes, respectively; and subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Chlorophyll fluorescence (CF) test

Fluorescence measurements, *Fo* and *Fm*, were taken from the most recent fully-expanded leaf on five plants of each of the ten cabbage lines, using a portable Photosynthetic Yield Analyser (Mini Pam; Heinz Walz GmbH, Effeltrich, Germany) after the plants had been dark-adapted using leaf-clips for 30 min. The five plants of each line had been arranged in a completely randomised design. Measurements of *Fo* and *Fm* were made on two occasions: (1) after the plants had been stressed at 30° – 45°C for 3 h (T2), achieved by closing the glasshouse ventilators on a hot Summer day; and (2) early the next morning when the plants were not under any stress, when the temperature was between 23º – 26ºC (T1). Values of *Fv* were calculated as the difference between *Fm* and *Fo* on each plant, and the *Fv/Fm* ratio was then determined for each temperature regime.

Analysis of variance was used to determine cultivar variations in RI and in the CF parameters, *Fo*, *Fv* and *Fv/Fm*, using the Genstat statistical package (VSN International, Hemel Hempstead, UK). The values for *Fv* and *Fo* were transformed (log_{10}) to normalise the error distributions before analysis.

RESULTS

RI values did not show any significant differences between lines (Table I), despite the fact that RI values ranged from 35.7 ('HRI 005237') to 68.0 ('HRI 009617'). The variability in RI within each line was high (CV = 28.3%), making it difficult to detect any differences between the ten lines in response to heat stress.

There were significant differences ($P < 0.01$) between stressed (T2) and non-stressed (T1) treatments for *Fo*, *Fv*, and the *Fv/Fm* ratio, and interactions between stress and line (stress × line interaction: $P = 0.019, 0.012, 0.062$,

TABLE I

Mean values (%) for relative injury (RI) in ten lines of cabbage treated at 50ºC for 15 min

Cabbage Line	RI (%)
'HRI 002605'	60.3*
'HRI 003202'	64.0
'HRI 003720'	54.7
'HRI 005237'	35.7
'HRI 006556'	50.1
'HRI 007827'	60.2
'HRI 009617'	68.0
'HRI 009837'	54.5
'HRI 011446'	64.1
'HRI 013011'	40.9

*n = 40; df = 30; Standard error of the difference (SED) = 11.05.

for *Fo*, *Fv*, and *Fv/Fm*, respectively) indicating that the response to stress differed between lines. This can be seen in Figure 1, which shows the pattern of all three fluorescence parameters in the ten lines of cabbage at T1 and T2. Generally, at T2, *Fo* values increased, while *Fv* and *Fv/Fm* decreased relative to the corresponding values at T1. The *Fv/Fm* ratios under non-stressed conditions were relatively constant, ranging from 0.83 – 0.86, with an average of 0.84. The greatest decreases in *Fv*, and in the *Fv/Fm* ratios of stressed plants were shown by 'HRI 002605', 'HRI 003202', and 'HRI 007827', indicating their higher susceptibility to heat; whereas 'HRI 013011', 'HRI 005237', and 'HRI 006556' showed smaller decreases in *Fv/Fm*. For two lines ('HRI 009837' and 'HRI 013011') *Fo* appeared to decrease under stressed conditions.

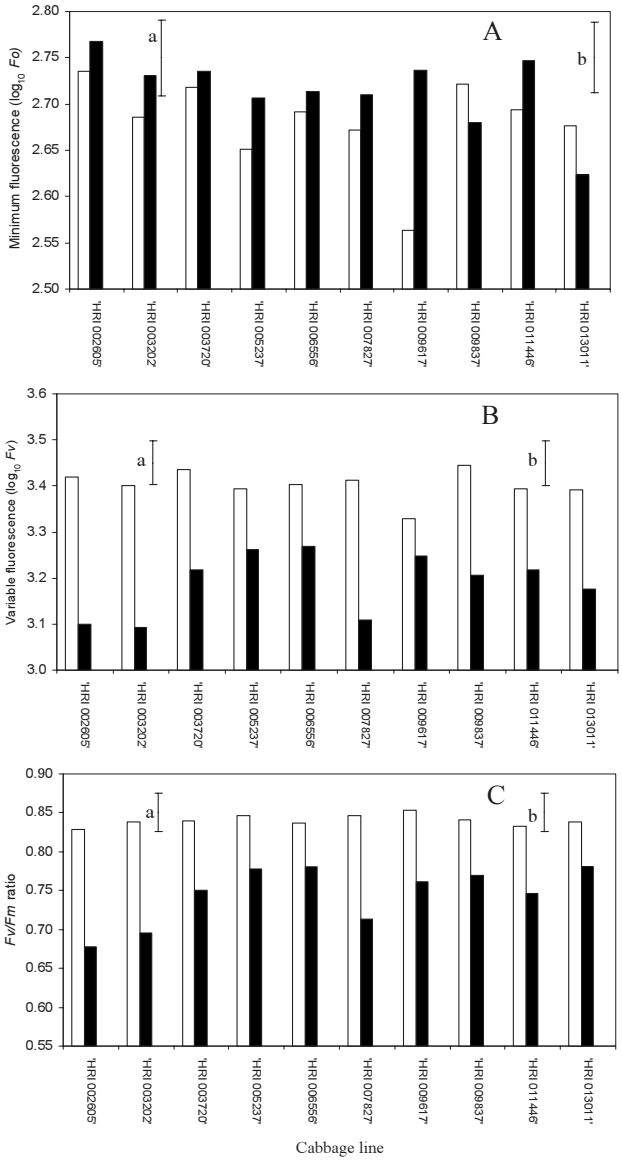

FIG. 1
Comparisons of minimum fluorescence (*Fo*; Panel A), variable fluorescence (*Fv*; Panel B), and the *Fv*/*Fm* ratios (Panel C) measured at 23º – 26ºC (T1; □) and at 35º – 40ºC (T2; ■) in ten varieties of cabbage. Error bars represent two standard errors of the difference (a compares means between different varieties, and b compares the T1 and T2 means for the same variety.) n = 100; df within a variety = 40, and between varieties = 80.

There was no relationship between RI and the three fluorescence parameters at T1. However, at T2, there were significant relationships ($P < 0.05$) between RI and *Fo*, and between RI and *Fv/Fm*, and a suggestion of a relationship ($P = 0.092$) between RI and *Fv* (Figure 2). Generally, RI increased as *Fv* and *Fv/Fm* decreased, and as *Fo* increased.

DISCUSSION

Only the most recent, fully-expanded leaves were used for the determination of RI, because it has been reported that genotypic differences in membrane thermostability were greatest in newly-developed leaves (Martineau *et al*., 1979). The scale of within-line variation observed in RI indicated that differences between lines would have to be larger than those observed in this study (Table I) to be detected. Similarly, Martineau *et al*. (1979) also found large plant-to-plant variation in RI measurements in four soybean (*Glycine max* L.) cultivars. It seems that RI would only be useful as a screening tool for heat tolerance in cabbage lines that exhibit extreme heat sensitivities, as reported by Chauhan and Senboku (1996). They found differences in RI between the heat-tolerant line 'Sousyu' and the heat-susceptible line 'YR Kishun'. It appears that the ten lines in the present study

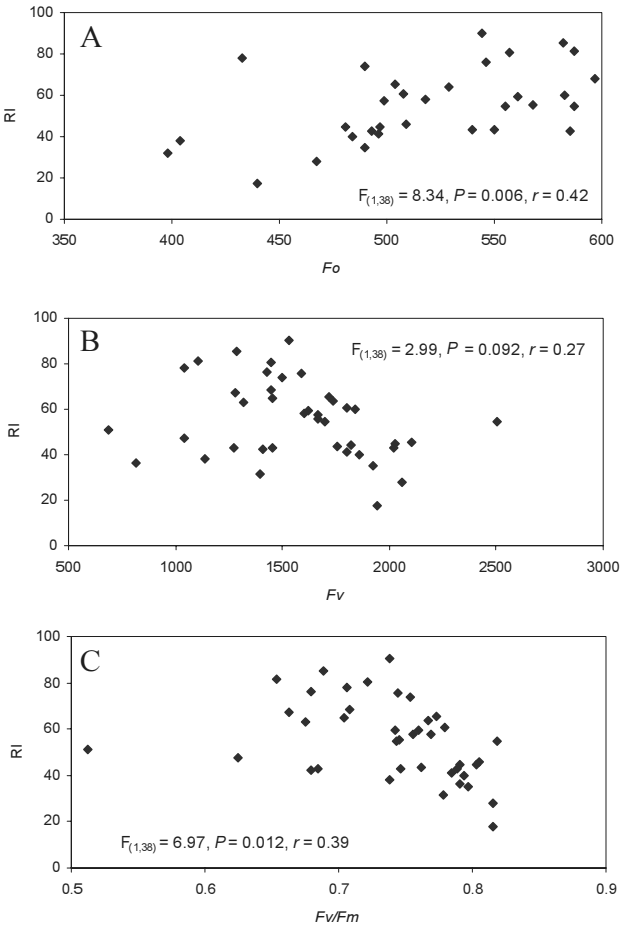

FIG. 2
Relationship between Relative Injury (RI) and the fluorescence parameters *Fo* (Panel A), *Fv* (Panel B), and the *Fv*/*Fm* ratio (Panel C) under stress temperature conditions (36º – 40ºC). *Fo* = minimum fluorescence; *Fv* = variable fluorescence; *Fm* = maximum fluorescence.

did not exhibit extreme heat tolerance, therefore the differences in CMT between the lines may be too small to detect. Techniques to reduce variability in measurements within lines are therefore needed to use CMT in such cabbages. From the coefficient of variation and the standard error of the data, the minimum number of replications needed to detect differences of the magnitude observed between the cabbage lines at the 5% significance level, with a power of 0.90, using a two-sided test, was calculated to be six. Therefore, if CMT is to be used to screen cabbage lines for heat tolerance in future, a minimum of six replicates should be used.

The differences between stressed and non-stressed plants, and their interaction for the CF parameters *Fv*, *Fo*, and the *Fv/Fm* ratio (Figure 1), showed how variable the ten cabbage lines were, and the different values gave an indication that there were some physiological differences between lines. *Fo* represents the level of fluorescence when the primary quinone electron acceptors of PSII are maximally oxidised (PSII centres are open). *Fm* represents the level of fluorescence when the primary quinone electron acceptors of PSII are maximally reduced (PSII centres are closed); whereas *Fv* demonstrates the ability of PSII to perform primary photochemistry (Baker and Rosenqvist, 2004). The *Fv/Fm* ratios at T1 gave a consistent average value of 0.84 for all cabbage lines (Figure 2C); whereas, at T2, the *Fv/Fm* ratio was lower than 0.84 for all lines. These values agree with previous literature (Bjorkman and Demmig, 1987; Johnson *et al*., 1993; Maxwell and Johnson, 2000) which indicated that optimum *Fv/Fm* values of approx. 0.83 were recorded for most plant species, and that, when heat-stressed, susceptible lines had much reduced *Fv/Fm* ratios. The dark-adapted values of *Fv/Fm* reflect the potential quantum efficiency of PSII and are used as an indicator of plant photosynthetic performance (Maxwell and Johnson, 2000). They also give the efficiency with which light (at low irradiance) is used (Chauhan and Senboku, 1996). Therefore, the extent of the decrease in the *Fv/Fm* ratio from 0.84, in response to heat stress, may be used to screen cabbages for heat tolerance.

The pattern of CF parameters here (Figure 1) confirmed earlier reports (Baker and Rosenqvist, 2004; Maxwell and Johnson, 2000) that, under stress, *Fo* increases, while *Fv* and *Fv/Fm* decrease. However, in the present study, *Fo* was not consistent for all cabbage lines (Figure 1). An increase in *Fo* may therefore not be a reliable indicator of heat tolerance for all lines of cabbage. Sipos and Prange (1986) also observed significant differences in *Fv* and *Fv/Fm*, but not in *Fo*, in ten lines of potato (*Solanum tuberosum* L.) showing different heat tolerance. Differences in *Fv* and *Fv/Fm* between cabbage lines at T2 revealed that these CF parameters are more sensitive indicators of heat tolerance in cabbage than RI, and hence could be used for screening cabbages for growth at high temperatures. This supports earlier literature in which Bjorkman *et al*. (1980) reported that the thylakoid membrane (which contains PSII) is more sensitive to heat than the cell membrane. The large decreases in both *Fv* and *Fv/Fm* values at T2 for lines 'HRI 002605', 'HRI 003202', and 'HRI 007827', and the smaller decreases in lines 'HRI 013011', 'HRI 005237', and 'HRI 006556', from those obtained at T1, indicate high susceptibility and high tolerance to heat, respectively. The four remaining lines can be said to be intermediate between these two groups in their tolerance of heat.

The fact that no relationships were detected between RI and the CF parameters measured at T1, whereas, at T2, there were some significant relationships (Figure 2), suggests that parameters measured under stress temperatures are more important in determining heat tolerance and that, at high temperatures, cell membranes and thylakoid membranes (PSII) are damaged simultaneously. These results also indicate that the thylakoid membrane (PSII) is more affected by heat than the cell membrane, as reflected by the decreases in fluorescence parameters (*Fv* and *Fv/Fm*) at T2. In general, the relationships at T2 revealed that RI increased as *Fv* and *Fv/Fm* decreased, and as *Fo* increased. This agrees with earlier work which showed that thermal damage to cell membranes was characterised by a marked increase in RI (Yeh and Hsu, 2004), whereas damage to thylakoid membranes (PSII) was characterised by a marked increase in *Fo* and a decrease in *Fv* and the *Fv/Fm* ratio (Hansatech, 1996; Sipos and Prange, 1986; Maxwell and Johnson, 2000).

CONCLUSION

Chlorophyll fluorescence parameters, especially *Fv* and *Fv/Fm*, were found to be more sensitive for screening cabbages for heat tolerance than RI, an index of CMT. Values of *Fv* and the *Fv/Fm* ratio of stressed plants showed a marked decrease for lines 'HRI 002605', 'HRI 003202', and 'HRI 007827', indicating higher susceptibility to heat; whereas the corresponding values for lines 'HRI 013011', 'HRI 005237', and 'HRI 006556' indicated a higher tolerance to temperatures commonly experienced in the tropics. A relationship ($P < 0.05$) between the RI and *Fo* and *Fv/Fm* existed only under stress conditions (35° – 40°C), which suggests that such parameters, measured at stress temperatures, are more important for determining heat tolerance.

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Corrigendum

YUNUSA, I. A. M., ZEPPEL, M. J. B. and NUBERG, I. K. (2008). Water-use efficiency reflects management practices in Australian olive groves. *Journal of Horticultural Science & Biotechnology*, **83,** 232-238.

The scheme was implemented correctly, but there was an error, as presented, in Eq. 2. The correct notation is as follows:

$$T = \frac{\rho Cp D g_c}{\lambda \gamma} K$$

where ρ is the density of air (kg m^{-3}), Cp is the specific heat of air, γ is the psychrometric constant, D is the vapour pressure deficit (kPa), λ is the latent heat of vaporisation, g_c is the canopy conductance that was obtained as the product of LAI and g_s, and K is the duration under consideration, which is daylight hours (in s) for the daily time-scale.