ミズナ(Brassica rapa L.)および九条ネギ(Allium fistulosum L.)幼根細胞の高温ストレスに対する2, 3, 5-triphenyltetrazolium chloride (TTC)染色の減少

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Studies on the reduction of 2,3,5-tripheniltetrazolium chloride (TTC) as a viability assay for Mizuna (*Brassica rapa* L.) and Welsh Onion (*Allium fistulosum* L.) Radicle Cellular Heat Stress

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Summary

To determine the conditions needed for a stable assay to evaluate thermo-tolerance of radicles in Mizuna and welsh onion seedlings, procedures of applying heat stress treatments were investigated by measuring the 2,3,5 - triphenyltetrazolium chloride (TTC) staining intensity of radicles in 2 day old Mizuna and 4 day old welsh onion germinated at 20°C and then subjected to 2-hour heat treatments at a range of temperatures.

When seedlings were treated by applying heat stress at 37, 40, 43, 45, 48 and 50°C with 100% relative humidity for 2 hr, the TTC staining was inhibited by the heat stress at 43°C and higher temperatures in 2 day old seedlings of Mizuna, and at 50°C in 4 day old seedlings of welsh onion.

Keywords : Mizuna, welsh onion, heat stress, radicle, thermo-tolerance, TTC staining

I Introduction

1. Global warming affects vegetable production in Kyoto

Heat stress due to high ambient temperatures is a serious threat to crop production globally, especially in temperate and tropical areas of the world (Hall, 2001).

Climate models project an increase in the surface temperature of the Earth by 1 to 11°C by 2100, because of greenhouse gas effects (Houghton et al., 2001, Stainforth et al., 2005). Additionally, short episodes of extreme climatic events including low and high temperatures are predicted to occur more frequently in the near future (Meehl and Tebaldi, 2004).

The rate of temperature rise in Western Japan, which includes be greater than the global average (Japan Meteorological Agency, 2009)

Recent high-temperature climatic events have reduced vegetable yield and quality in Kyoto prefecture.

Mizuna (*Brassica rapa* L.) and Kujo type welsh onion (*Allium fistulosum* L.) are major vegetable crops in Kyoto prefecture. Under high-temperature stress, the two vegetable species show responses such as poor germination, low yield, and poor quality. Therefore, to cope with high temperatures, plant physiological studies, heat tolerant cultivars and cultural methods which reduce heat stress are required for Mizuna and Kujo welsh onion.

2. Heat injuries in plants

Under extreme high temperatures, severe cellular injury and even cell death may occur within minutes, which could be attributed to a catastrophic collapse of cellular organization (Schöffl et al., 1999). At moderately high temperatures, injuries or death may occur only after long-term exposure. Direct injuries due to high temperatures include protein denaturation and aggregation, and increased fluidity of membrane lipids. Indirect or slower heat injuries include

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inactivation of enzymes in chloroplasts and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity (Howarth, 2005). These injuries eventually lead to starvation, inhibition of growth, reduced iron flux, production of toxic compounds and reactive oxygen species (ROS) (Schöffl et al., 1999; Liu and Huang 2000; Howarth, 2005).

3. Heat tolerance differs for plant species, genotypes and growth stages

A threshold temperature refers to a value of daily mean temperature at which a detectable reduction in growth begins. Upper and lower developmental threshold temperatures have been determined for many plant species through controlled incubator, phytotron and field experiments. An upper developmental threshold is the temperature above which growth and development cease. Upper threshold temperatures also differ for different plant species and genotypes within species. Moreover, the threshold temperatures are affected by the growth stages in a particular genotype. Upper threshold temperatures for some major crop species are displayed in Table 1. High temperature sensitivity is particularly important even in temperate areas affected by global warming, as heat stress may become a major limiting factor for field crop production.

4. Assays for studying the effect of heat stress

There are several assays to estimate threshold temperatures. In these assays, 2,3,5 - triphenyltetrazolium chloride (TTC) staining has been used to measure viability of several plant tissues after applying stress treatments (Purcell and Young, 1963; Caldwell, 1993; Dan and Imada, 2002). Living plant cells reduce TTC in their mitochondria, producing carmine-red to cherry-red formazan, but dead cells do not. Tissues can then be visually assessed for viability by subjectively classifying tissues as living or dead based on the appearance of the red colour (Roberts, 1951; Purcell and Young, 1963; Towill and Mazur, 1975; Yaklich and Kulik, 1979).

5. Aim of this study

Until now, physiological effects of high temperature stress have not been studied in two local cultivars of Mizuna and Kujo type welsh onion. First of all, we applied TTC staining on Mizuna and welsh onion in the early seedling stages and determined the conditions of the assay. Then we tried to detect threshold high temperatures by using short term stress treatments.

Table 1. Threshold high temperatures for some crop plants

Vegetable plants	Threshold temperature (°C)	Growth stage	References
Brassica	29	Flowering	Morrison and Stewart (2002)
Allium altissimum	30	Emergence	Rezaie et al. (2012)
Solanum lycopersicum (Tomato)	30	Emergence	Camejo et al. (2005)
Arachis hypogea L. (Groundnut)	34	Pollen production	Vara Prasad et al. (2000)
Pennisetum glaucum (Pearl millet)	35	Seedling	Ashraf and Hafeez (2004)
Zea may s L. (Corn)	38	Grain filling	Thompson (1986)
Gossypium hirsutum L. (Cotton)	45	Reproductive	Rehman et al. (2004)

II Materials and Methods

1. Seed source

Seeds of Mizuna (*Brassica rapa* L.) cv. 'Jyonan-sensuji' were obtained from Ishihara Seeds Ltd., Kyoto, Japan and those of welsh onion (*Allium fistulosum* L.) cv. 'Asagikei-kujo' were obtained from Takii & Co., Ltd., Kyoto, Japan. These two cultivars are standard for Mizuna and welsh onion production in Kyoto prefecture. The seeds of Mizuna and welsh onion were stored in closed plastic bags in a cold room at 5°C before the experiments.

2. Germination

Mizuna and welsh onion seeds were placed in plastic dishes (Iwaki Code 1020-100 ϕ = 100mm, Shizuoka, Japan) on two sheets of filter paper (Advantec No2, ϕ = 7cm, Tokyo, Japan) moistened with 3mL of distilled water. 49 seeds were distributed evenly within each dish. One dish was used as a plot and treatments were applied to three times. The dishes were covered with their lids by parafilm (Pechiney Plastic Packaging Company, Chicago, IL, U.S.A.) in order to keep 100% of relative humidity (RH) in a forced-air incubator.

The temperature in the incubator was maintained at 20°C during 2 days of emergence period and given 12,000 lux of continuous fluorescent light illumination for Mizuna.

As for welsh onion, the incubator was maintained at the same temperature as the Mizuna one, but given dark conditions for 3 days, then fluorescent light was turned on for one day, to provide the best lighting conditions for each species.

3. Heat stress treatments

The plastic dishes for Mizuna and welsh onion seedlings were moved into a convection oven (Sanyo) maintained at either 37, 40, 43, 45, 48 or 50°C for two hours of high temperature treatment. The convection oven was placed in a room at a temperature below 25°C to keep stable temperature stress in the convection oven without cooling function. So, the heat stress was always of only two hours duration. The same stress tests were done in three times for each species.

4. 2,3,5-triphenyltetrazolium chloride (TTC) staining

TTC was dissolved in 50mM of phosphate buffer (pH 7.5) to make 0.1 % (w/v) of solution.

After the heat treatments, 24 vigorous seedlings from each treatment dish were moved into 2ml of plastic tubes and 1.8 ml of the TTC solution was added to each tube. The seedlings were submerged for 15 hours at 25°C in darkness.

5. Survival confirmation tests after heat stress treatment

12 seedlings of Mizuna and welsh onion were given heat treatments at 43 and 50 °C, respectively. The procedures were same as the above-mentioned methods. Survival of the 12 seedling were observed after 2 and 3days culture at 20 °C under light condition for Mizuna and welsh onion, respectively.

6. Evaluation of the seedlings

The staining patterns of each seedling were recorded according to the following criteria: 0 = not well stained; 1 = proximal half of the radicle stained; 2= proximal three fourths of radicle stained; 3 = whole radicle well stained (Figure 1). Then staining reduction index (SRI) was calculated by following formula.

SRI = Σ (criteria × number of plants with each criteria)/total number of plants.

The temperature treatment criteria data from all experiments were subjected to the non-parametric Kruskal-Wallis test followed by the Steel-Dwass test for multiple comparison at the 0.05 significance level. In order to estimate reproducibility of each temperature treatment, statistical significance among three repetition tests of staining criteria in each temperature treatment were also examined using the Steel-Dwass test .

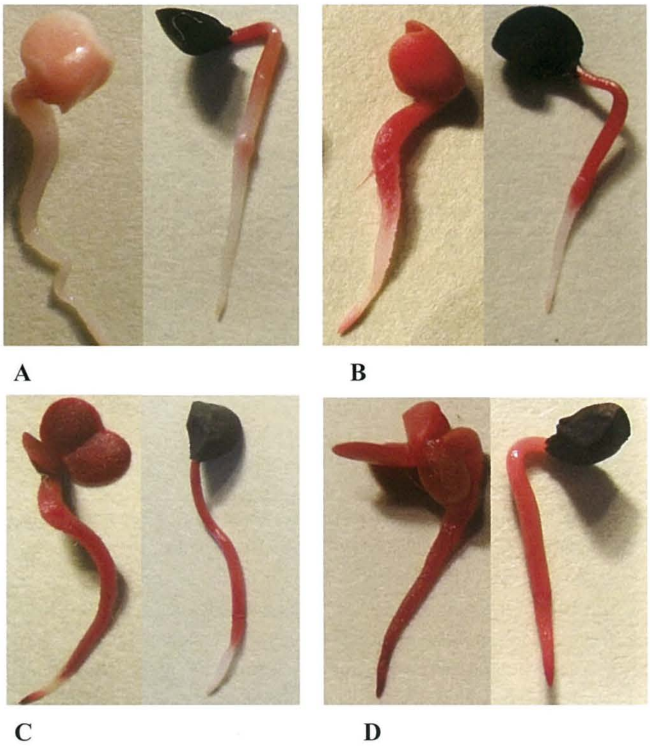

Fig 1. TTC staining of radicles of Mizuna 'Jonan-sensuji' (left) and welsh onion 'Asagikei-kujo' (right).

Criteria of the staining:

A, 0 = not well stained;

B, 1 = proximal half of the radicle stained;

C, 2= proximal three fourths of radicle stained;

D, 3 = whole radicle well stained

Ⅲ Result

Heat stress at 43°C and higher temperature treatments suppressed TTC staining in 2 day old Mizuna seedlings.

After 43, 45, 48 and 50°C treatments, the extent of TTC staining was significantly different from the lower temperature treatments, and no seedlings were well stained after the 50°C treatment. Statistical significance was found in three repetition tests of the 43 and 48 °C treatment (Table 2).

Unlike the staining condition in Mizuna seedlings, all seedlings of 4 day old welsh onion were well stained after 37, 40 and 43°C treatments, and most of them were also well stained after the 45°C treatment. The extent of the staining after the 48°C treatment was significantly lower than from the 37, 40 and 43°C treatments. However, most of the seedlings (88%) were also completely stained after 48°C (Table 3). Moreover, when single replicate scale of the gradient heat temperature treatments (37 to 50°C and control plots) were compared by statistical test, the extent of the staining after 48°C treatment was not significantly lower the staining extent in any replicate of the temperatures set (data not shown). However, the percentage of completely stained seedlings dropped greatly after 50°C treatment (Table 3), and the extent of the staining after 50°C was significantly lower than in 37, 40, 43, 45 and 48°C in any replicate of three times (data not shown).

As for the survival confirmation, all of the seedling survived after 2 and 3 days culture in Mizuna and welsh onion, respectively.

Table 2. The extent of TTC staining of 2 day old Mizuna seedlings after 2 hr of heat stress treatment at various temperatures

Treatment	Number of the seedlings in each criteria of TTC staining [1]				Rate of completely	Staining reduction
Temperature (°C)	3	2	1	0	staining seedlings	index (SRI) [2] [3] [4]
37	72	0	0	0	100%	3.00 [a]
40	69	3	0	0	96%	2.96 [a]
43	42	29	1	0	58%	2.57 [b*]
45	1	62	9	0	1%	1.89 [c]
48	0	6	59	7	0%	0.99 [d*]
50	0	0	0	72	0%	0.00 [e]
20 (Control)	72	0	0	0	100%	3.00 [a]

1) Total number of seedlings in each criteria in three repetiton tests.
Criteria : 0 = not well stained; 1 = proximal half of the radicle stained; 2= proximal three fourths of radicle stained;
3 = whole radicle well stained

2) SRI =Σ (criteria × number of plants with each criteria)/total number of plants.

3) Statistical significance of differences was tested in 7 temperature treatments by using the staining criteria scores.
Any two temperature treatments not followed by the sameletter are significantly different at $P \leq 0.05$ according to theKruskal-Wallis test followed by the Steel-Dwass test for multiple comparison.

4) Statistical significance of differences was tested among three repetition tests of the staining criteria in each treatment temperature.
* indicates significance at $P \leq 0.05$ according to the Kruskal-Wallis.

Table 3. The extent of TTC staining of 4 day old welsh onion onion seedlings after 2 hr of heat stress treatment at various temperatures

Treatment	Number of the seedlings in each criteria of TTC staining $^{1)}$				Rate of completely	Staining reduction
Temperature (°C)	3	2	1	0	staining seedlings	index (SRI) $^{2)\,3)\,4)}$
37	72	0	0	0	100%	3.00 ^{a}
40	72	0	0	0	100%	3.00 ^{a}
43	72	0	0	0	100%	3.00 ^{a}
45	71	1	0	0	99%	2.99 ^{ab}
48	63	7	2	0	88%	2.85 ^{b}
50	8	31	32	1	11%	1.64 ^{c*}
20(Control)	72	0	0	0	100%	3.00 ^{a}

1) Total number of seedlings in each criteria in three repetiton tests.
Criteria : 0 = not well stained; 1 = proximal half of the radicle stained; 2= proximal three fourths of radicle stained;
3 = whole radicle well stained

2) SRI =Σ (criteria × number of plants with each criteria)/total number of plants.

3) Statistical significance of differences was tested in 7 temperature treatments by using the staining criteria scores.
Any two temperature treatments not followed by the sameletter are significantly different at $P \le 0.05$ according to the Kruskal-Wallis test followed by the Steel-Dwass test for multiple comparison.

4) Statistical significance of differences was tested among three repetition tests of the staining criteria in each treatment temperature.
* indicates significance at $P \le 0.05$ according to the Kruskal-Wallis.

Ⅳ Discussion

1. Threshold temperature detection and its stability

In these experiments, we tried to detect threshold temperatures for short term heat stress that affect viability of Mizuna and welsh onion seedlings. The threshold temperatures under our test conditions were considered to be 43°C in Mizuna and 50°C in welsh onion from the results of three repetition tests of the assay in this study. Then, we confirmed the survival of the seedlings at the threshold.

However, the extents of TTC staining results at 43°C and 48°C treatments were somewhat unstable and significantly different among three repetition tests in each temperature treatment at 43°C and 48°C, respectively.

The instability of this assay may be considered to be due to the following three reasons.

The first candidate is the condition of the seedlings. In this assay, we selected 24 rather vigorous seedlings from 48 ones. Nevertheless, the nutrient condition of each seedling may be uneven. Dan et al., (2002) reported a similar experiment with cucumber. In this test, they use 1% of sucrose as supplemental nutrient.

The second candidate is the genetically uneven background. Mizuna is not self-compatible, nor is welsh onion. So, most of the seedlings of Mizuna and welsh onion were not genetically identical within a particular cultivar.

Thirdly, instability of the heat stress treatment is considered. Although the convection oven used for heat stress in this experiment is controlled by PID (Proportional Integral Derivative) controller, temperature accuracy is ±1°C by the specifications of this equipment. Further study is required on each of these factors.

Notwithstanding, the procedure of this study has been effective to separate threshold temperatures on an experimental scale using three repetition tests.

2. Remarks about observations on staining

The radicles of the seedlings were very small and thin. Therefore, the radicles were easily dried out, once the seedlings were removed from the TTC solution. It became difficult to distinguish the staining condition by using dried seedlings. So, the pictures of stained seedlings should be taken to magnify the radicles and record the staining condition before they have dried out.

In addition, assay procedures such as incubation (temperature, light intensity, RH and duration), heat stress regimes and staining conditions should be strictly observed, or the experimental result is not replicable. Before we decided on the conditions of this assay, we tried several incubation terms, light conditions and RH. Then we confirmed that different assay conditions resulted in different extents of staining, even if there was only a minor change of procedure (data not shown).

3. Utilization of this assay

To cope with high temperatures, selecting heat tolerant cultivars is one of the important strategies. By using this assay, it might be possible to assess short term heat tolerance of cultivars in the early seedling stage.

As for other approaches, there is plant acclimation to heat stress (Chen et al., 1982). Immediately after exposure to heat stress under threshold temperature, changes occur at the molecular level altering the expression of genes and accumulation of transcripts, thereby leading to the synthesis of stress-related proteins as a stress tolerance strategy (Dan and Imada, 2002; Iba, 2002). Expression of heat shock proteins (HSPs) is known to be an important adaptive strategy in this regard (Feder and Hoffman, 1999). The HSPs have chaperone-like functions and are involved in signal transduction during heat stress (Schöffl et al., 1999). The tolerance conferred by HSPs results in improved physiological phenomena such as photosynthesis, assimilate partitioning, water and nutrient use efficiency, membrane stability and disease tolerance (Camejo et al., 2005; Ahn and Zimmerman, 2006; Momcilovic and Ristic, 2007; Widiastuti et al., 2013). Such improvements make plant growth and development possible under heat stress. However, not all genotypes within species have similar capabilities in coping with heat stress. There exists tremendous variation within and between species, providing opportunities to improve crop heat-stress tolerance through genetic means (Ehlers and Hall, 1998; Camejo et al., 2005). The assay of this study might be used to estimate the extent of particular genotypes' acclimation to heat stress, if the assay were to be carried out after pre-incubation just below the temperature of the thresholds.

4. Conclusion

In this study, we determined an assay for assessing viability and detecting threshold temperatures at an early stage of growth in two crop species. This assay can be a useful tool for evaluating heat tolerance in cultivars and for studying plant acclimation to heat stress in Mizuna and welsh onion.

V Acknowledgements

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VI Literature Cited

(1) Ahn, Y.J. and J.L. Zimmerman (2006) Introduction of the carrot HSP17.7 into potato (*Solanum tuberosum* L.) enhances cellular membrane stability and tuberization in vitro, Plant Cell Environ., 29:95-104

(2) Ashraf, M. and M. Hafeez (2004) Thermotolerance of pearl millet and maize at early growth stages: growth and nutrient relations, Biol. Plant. 48: 81–86

(3) Caldwell, C.R. (1993) Estimation and analysis of cucumber (Cucumis sativus L.) leaf cellular heat sensitivity, Plant Physiol., 101: 939-945

(4) Camejo, D., P. Rodríguez, M.A. Morales, J.M. Dell'Amico, A. Torrecillas and J.J. Alarcón (2005) High temperature effects on photosynthetic activity of two tomato cultivars with different heat susceptibility, J. Plant Physiol. 162: 281–289

(5) Chen, H. H., Z. Y. Shen and P. H. Li (1982) Adaptability of crop plants to high temperature stress, Crop Sci., 22: 719-725

(6) Dan, K and S. Imada (2002) Effect of high temperature on viability and growth of radicles in cucumber seedlings (In Japanese with English summary), J. Japan. Soc. Hort. Sci., 71(6): 805-811

(7) Ehlers. J.D. and A.E. Hall (1998) Heat tolerance of contrasting cowpea lines in short and long days, Field Crops Res. 55: 11-21

(8) Feder, M.E. and G.E. Hoffman (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology, Annu. Rev. Physiol. 61: 243-282

(9) Hall, A.E. (2001) Crop responses to environment. CRC Press LLC, Boca Raton, Florida

(10) Houghton, J.T., Y. Ding, D. J. Griggs, M. Noguer, P. J. van der Linden, X. Dai, K. Maskell, and C. A. Johnson (2001) Climate change 2001: The scientific basis contribution of working group I to the third assessment report of the intergovernmental panel on climate change, Cambridge, University Press, New York, pp 881

(11) Howarth, C.J., 2005. Genetic improvements of tolerance to high temperature, In: (Ashraf, M., Harris, P.J.C. eds.), Abiotic stresses: Plant resistance through breeding and molecular approaches, Howarth Press Inc., New York

(12) Iba, K. (2002) Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance, Annu. Rev. Plant Biol. 53: 225–245

(13) Japan Meteorological Agency, Ministry of the Environment, Ministry of Education, Culture, Sports, Science and Technology (2009) Climate change and its impacts in Japan: Synthesis report on observations, projections, and impact assessments of climate change, https://www.env.go.jp/en/earth/cc/report_impacts.pdf

(14) Liu, X. and B. Huang (2000) Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass, Crop Sci., 40: 503-510

(15) Meehl, T.G.A. and C. Tebaldi (2004) More intense, more frequent, and longer lasting heat waves in the 21st century, Science 305: 994–997

(16) Momcilovic, I. and Z. Ristic (2007) Expression of chloroplast protein synthesis elongation factor, EF-Tu, in two lines of maize with contrasting tolerance to heat stress during early stages of plant development, J. Plant Physiol. 164: 90-99

(17) Morrison, M.J. and D.W. Stewart (2002) Heat stress during flowering in summer brassica, Crop Sci. 42: 797–803

(18) Purcell. A.E. and R.H. Young. (1963) The use of tetrazolium in assessing freeze damage in Citrus trees, Proc. Amer. Soc. Hort. Sci. 83: 352-358

(19) Rehman, H., S.A. Malik and M. Saleem (2004) Heat tolerance of upland cotton during the fruiting stage evaluated during cellular membrane thermostability, Field Crops Res. 85: 149–158

(20) Rezaie E.E., H. Mansoori, M. Kafi and M. Bannayan (2012) Emergence response of Persian shallot (*Allium altissimum*) to temperature, Afr. J. Agric. Res. 7(38): 5312-5316

(21) Roberts, L. W. (1951) Survey of factors responsible for reduction of 2,3,5- triphenyltetrazolium chloride in plant meristem. Science, 113: 692-693.

(22) Schöffl, F., R. Prandl and A. Reindl (1999) Molecular responses to heat stress. In: (Shinozaki, K., Yamaguchi-Shinozaki, K. eds.), Molecular responses to cold, drought, heat and salt stress in higher plants, R.G. Landes Co., Austin, Texas, pp 81–98

(23) Stainforth, D.A., T. Aina, C. Christensen, M. Collins, N. Faull, D.J. Frame, J.A. Kettleborough, S. Knight, A. Martin, J.M. Murphy, C. Piani, D. Sexton, L.A. Smith, R.A. Spicer, A.J. Thorpe and M.R. Allen (2005) Uncertainty in predictions of the climate response to rising levels of greenhouse gases, Nature 433: 403–406

(24) Thompson, L.M. (1986) Climatic change, weather variability and corn production, Agron. J. 78: 649–653

(25) Towill, L. and P. Mazur (1975) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can. J. Bot. 53: 1097-1102

(26) Vara Prasad, P.V., P.Q. Craufurd, R.J. Summerfield and T.R. Wheeler (2000) Effect of short epidosed of heat stress on flower production and fruit set of groundnut (*Arachis hypogea* L.), J. Exp. Bot. 51: 777–784

(27) Widiastuti, A., M. Yoshino, M. Hasegawa, Y. Nitta and T. Sato (2013) Induction of disease resistance against Botrytis cinerea by heat shock treatment in melon (*Cucumis melo* L.), Physiol. Mol. Plant Pathol. 82:52-55

(28) Yaklich, R. W. and M. M. Kulik (1979) Evaluation of vigor tests in soybean seeds: relationship of the standard germination test, seedling vigor classification, seedling length, and tetrazolium staining to field performance, Crop Sci. 19: 247-252