



In vitro evaluation of osmotic stress tolerance using a novel root recovery assay

Maruyama, Hideki ; Koyama, Ryohei ; Oi, Takeru ; Yagi, Masafumi ;
Takeda, Migiwa ; Kanechi, Michio ; Inagaki, Noboru ; Uno, Yuichi

(Citation)

Plant Cell, Tissue and Organ Culture, 95(1):101-106

(Issue Date)

2008-07-22

(Resource Type)

journal article

(Version)

Accepted Manuscript

(URL)

<https://hdl.handle.net/20.500.14094/90000934>



Title page

Title

In vitro evaluation of osmotic stress tolerance using a novel root recovery assay

Authors

Hideki Maruyama^{1§}, Ryohei Koyama^{2§}, Takeru Oi², Masafumi Yagi^{2†}, Migiwa Takeda^{2‡},
Michio Kanechi¹, Noboru Inagaki¹ and Yuichi Uno^{1*}

Affiliations

¹Plant Resource Science, Graduate School of Agricultural Science, KOBE University

²Graduate School of Science and Technology, KOBE University

ROKKO, KOBE 657-8501, JAPAN

([†]Present Address; National Institute of Floricultural Science, Tsukuba 305–8519, JAPAN)

([‡]Present Address; Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, JAPAN)

(*requests for offprints; Fax: +81-78-803-5832; E-mail: yuno@kobe-u.ac.jp)

([§]contributed equally to this work)

Complete correspondence address to which the proofs should be sent

Yuichi Uno

Plant Resource Science, Graduate School of Agricultural Science, KOBE University

ROKKO, KOBE 657-8501, JAPAN

Style of papers

Research notes

Keywords: drought stress, growth, lettuce, root-bending assay, salinity stress, survival

Abstract

We established a novel *in vitro* method, termed the root recovery assay, to evaluate the survival under osmotic stress of lettuce (*Lactuca sativa* L.) seedlings. Under salinity and drought stress, combination of the root-bending assay and root recovery assay showed the same trends in dry weight and survival rate as a hydroponic culture. Both *in vitro* assays and hydroponics ranked the three lettuce cultivars in the same order of drought tolerance. The root-bending assay evaluated the plant's growth and the root recovery assay indicated the plant's survival. In addition, the combined assay required less space and approximately half the time period compared with the hydroponic culture. These results suggested that application of the root-bending and root recovery assay should be a rapid and space-saving method with which to evaluate the osmotic stress tolerance of lettuce from both growth and survival standpoints.

Abbreviations: cv. – cultivar; MS – Murashige and Skoog; PEG – polyethylene glycol

Introduction

Osmotic stresses, such as high salinity and drought, negatively influence crop yield. Evaluation of stress tolerance is important in order to select an appropriate variety to cultivate in unfavorable conditions. Although much investigative effort has been directed toward this goal, most of the studies have evaluated the stress tolerance of plants by measuring either growth or survival. Slower growth induced by abiotic stress

is an adaptive feature for plant survival because it allows plants to rely on multiple resources to combat stress (Zhu, 2001). Furthermore, a general trade-off function between drought tolerance and maximum plant growth rate was assumed by a mathematical model (Sambatti and Caylor, 2007). In fact, the constitutive overexpression of the stress-related *DREB/CBF* gene has been shown to cause not only stress tolerance but also growth retardation of transgenic plants (Liu et al., 1998). Assessment using either a growth or survival index is probably only a partial evaluation of the stress tolerance. Another problem with the evaluation of stress tolerance is that a classical method such as hydroponic culture requires a long time period and a large area. The root-bending assay was established for screening cadmium-sensitive mutants in *Arabidopsis* (Howden and Cobbett, 1992), and subsequently was applied to screen mutants for salt tolerance (Wu et al., 1996). Recently, it was demonstrated that this assay was suitable for screening mutants related to proton rhizotoxicity (Iuchi et al., 2007). This method saves a great deal of space and time through the utilization of seedlings *in vitro* for the test. Root bending is supposed to reflect plant growth (Howden and Cobbett, 1992; Wu et al., 1996). For the reason mentioned above, the novel root recovery assay was adopted in the present study to evaluate plant survival under osmotic stress. We examined the responses of lettuce (*Lactuca sativa* L.), which is one of the most-consumed vegetables in the world. Lettuce is classified as a moderately sensitive crop with a threshold salinity level of 1.3 dS m^{-1} at which no yield loss occurs ($\approx 11 \text{ mM NaCl}$; Ashraf, 1994) and a 50% reduction in yield occurs at 5.2 dS m^{-1} ($\approx 55 \text{ mM NaCl}$; Orcutt and Nilsen, 2000). Improvement of the osmotic stress tolerance of lettuce is desirable for the breeding of new cultivars. The aim of this work was to study the applicability of combination of the root-bending assay and the root recovery assay to provide an index of osmotic stress tolerance in lettuce.

Materials and Methods

Seeds of lettuce cv. Okayama-Saradana (Takii Seed Co., Japan) were germinated in LC-1 Hortcube growing medium (SMITHERS-OASIS, Ohio, US) and irrigated with 0.1% (v/v) Hyponex (N:P:K = 6:10:5, Hyponex, Osaka, Japan). After 25 d seedlings were transferred to a hydroponic system. After 5 d acclimatization, seedlings were treated with nutrient solution containing 0–200 mM NaCl. Drought stress was imposed by withholding water from seedlings with approximately seven true leaves, that were germinated and grown in a vermiculite bed. The relative survival percentage and dry mass were determined after 16 d of NaCl treatment or every other day until 14 d of dehydration stress. The dry weight was measured after drying the plantlets in an oven at 80 °C for a minimum of 48 h. The relative dry weight (RDW) was calculated with the following formula: $RDW = (\text{dry weight of stressed plants})/(\text{dry weight of control plants}) \times 100$. The death of a plant was determined by observation of dessication or browning of the apical bud.

The modified root-bending assay (Howden and Cobbett, 1992; Wu et al., 1996) and the root recovery assay were carried out as follows. Five-day-old seedlings germinated on Murashige and Skoog's basal medium (MS) (Murashige and Skoog, 1962) at pH 5.7–5.8 supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar were placed upside down on the assay medium (Figure 1A). The assay medium comprised the MS medium modified by containing 1.0% (w/v) agar (Wako Pure Chemical Industries Ltd, Osaka, Japan) and either 0–600 mM NaCl or 0–15% (v/v) polyethylene glycol (PEG) #200. Seven replicate seedlings were assayed in each culture bottle (100×100×110 mm). If a seedling was resistant to the osmotic stress, its root bent due to

gravitropism (Figure 1A). After the root-bending assay, the seedlings were rehydrated by transferal to standard MS medium lacking NaCl or PEG (Figure 1B). The recovery of a seedling was judged to have occurred with the emergence of lateral roots. The root-bending and recovery ratios were calculated 7 and 14 d, respectively, after the assay began. Both the hydroponic and *in vitro* cultures were maintained at 22 °C under a 16 h photoperiod of approximately 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light. An additional two lettuce cultivars (cv. Marino and cv. Blizzard; Yokohama Nursery Co. Ltd, Japan) were used for comparison of drought stress tolerance in the *in vitro* assays and the hydroponic experiment.

Results and Discussion

In order to ascertain the appropriateness of the survival/death judgment for the plants grown hydroponically, a pilot experiment was conducted to evaluate the correlation between water content and defined survivability (data not shown). The water content of all of the withered plants was less than 50% with a maximum of 49.8%, and that of surviving plants was more than 60% with a minimum of 61.7%. In addition, none of the withered plants recovered in the rehydration treatment, whereas the non-withered plants did recover (data not shown). According to these results, the observation of desiccation or browning of the apical bud was an appropriate index of survivability.

Dry weight, which is the most common measure of plant growth, gradually declined with increasing duration of dehydration stress in the hydroponic culture (Figure 2A). In contrast, the survival rate suddenly declined after 10 d (Figure 2A). Similar results were obtained in the NaCl stress experiment. The growth inhibition

curve showed a gradual decrease with increasing NaCl concentrations ranging from 0 to 150 mM. In contrast, salinity of up to 100 mM NaCl did not affect the survival percentage, but NaCl concentrations exceeding 150 mM significantly reduced survival to less than 40% of the control (Figure 2B). The results of these experiments indicated that the assessment of osmotic stress tolerance can differ with measurement of either growth or survivability, as well as the timing of the measurement.

Zhu (2001) argued that the extent of salt or drought tolerance often appears to be inversely related to growth rate in nature. Taken together with our results, early growth retardation seems to be one of the strategies for survival under osmotic stress. It may be difficult for plants to maintain both growth and survivability concurrently when under stress. Therefore, prioritization of either trait is essential in the breeding of stress-tolerant crops. Maintenance of growth is an appropriate strategy in crop breeding to prevent a reduction in yield on arable land in response to a transient stress. For example, salt accumulation or spray strewn by a storm or typhoon in coastal areas, and a temporary water shortage resulting from abnormal weather might be classified as transient, because these moderate stresses can be avoided by artificial treatments, such as irrigation or overhead sprinkling with water. Early growth retardation will provide short-term relief until a remedy is applied or conditions return to normal. Prioritization of survivability of plants will be best suited for the development of crops to be grown in a severe environment, such as salt marshes, deserts, or arid regions. Prolonging the plant's life at the expense of reduced growth would contribute toward this objective.

The assessment of stress tolerance *in vitro* by combination of the root-bending assay and the root recovery assay generated similar results to the experiments on hydroponic cultures (Figure 3A and B). Both the root-bending and root recovery assays showed greater tolerance of seedlings *in vitro* than those of whole plants tested

hydroponically. This may be due to tissue specificity or environmental factors such as the high humidity in the culture bottle. Newmann (1995) mentioned that even though salinity can induce a rapid reduction in root growth, shoot growth decreases proportionally more than root growth. As observation of bending was a more simplified evaluation than measurement of dry weight, the root-bending curve had a steeper slope compared to the dry weight growth inhibition curve shown in Figure 2. The root-bending ratio, which is an indicator of growth sustainability under stress (Howden and Cobbett, 1992; Wu et al., 1996), was not affected at PEG concentrations up to 5.0% (≈ 280 mM), but declined with increasing PEG concentrations ranging from 5.0 to 12.0% (Figure 3A). Following transferal to an assay medium lacking PEG for rehydration, new lateral roots emerged in some seedlings that did not exhibit root bending, while other seedlings showed no change. These observations made it possible to determine either the survival or death of the plantlets. Although at 7.0 and 8.0% PEG the root-bending ratio was under 50%, the root recovery ratio was 100% (Figure 3A). Similarly, the root-bending ratio under salt stress was lower in comparison with the root recovery rate (Figure 3B). As the threshold NaCl concentration for survival was considered to be 400–500 mM, a further experiment was conducted to detect the effect of the stress period on the recovery ratio. NaCl stress treatment for 1 d resulted in higher root recovery ratios of 79% (400 mM NaCl), 9% (450 mM) and 0% (500 mM; data not shown) in comparison with those recorded after 14 d (Figure 3B). From these results, the root recovery index represents the survival of plants under osmotic stress.

In order to demonstrate the accuracy of the root-bending and recovery assays, the stress tolerances of three lettuce cultivars (cvs. Okayama-Saradana, Marino and Blizzard) were examined in both *in vitro* and hydroponic cultures. The root-bending and recovery ratios exhibited similar trends to the relative dry weight and the survival ratios

under drought stress in the hydroponic culture (data not shown). Table 1 summarizes the results for each ratio after 12 d of dehydration stress and for the 10% PEG treatment. These criteria were determined as the thresholds at which Okayama-Saradana showed about 50% survival or recovery ratios (Figures 2 and 3). With regard to the root-bending ratio, the tolerance of the cultivars was in the rank order Blizzard > Marino > Okayama-Saradana. The relative dry weight did not differ significantly between cultivars but showed the same order of tolerance as that estimated by the root-bending ratio. The cultivar order of tolerance for the survival indices differed from that of the growth indices, viz. Marino > Blizzard > Okayama-Saradana. This result supported the hypothesized relationship between early growth retardation and survivability of plants under drought stress as mentioned already. Although significant differences were found only in the root-bending and recovery assays ($P < 0.01$), this might reflect the more strict environmental control *in vitro*.

There are three parameters that might be used to assess the effect of salt on a particular species, namely survival, vegetative growth and seed production (Flowers and Yeo, 1989). At least growth and survival can be assessed by the root-bending and recovery assays. Moreover, a combination of these assays could shorten the experimental period required. The salinity and drought stress experiments with hydroponic cultures ran for 39–46 d for each entire experiment, whereas the *in vitro* assays needed only a 26 d period. Furthermore, *in vitro* assays require much less space and allow evaluation of many samples simultaneously. The assays can be utilized for basic estimation of tolerance differences among many cultivars, in transgenic studies, and for high-throughput screening. We concluded that combination of the root-bending and root recovery assays are a rapid and space-saving method to evaluate the osmotic stress tolerance of lettuce from both growth and survival standpoints.

Acknowledgement

This work was supported in part by a grant from the Salt Science Research Foundation No. 0513.

References

- Ashraf M (1994) Breeding for salinity tolerance in plants. *Crit Rev Plant Sci* 13: 17–42
- Flowers TJ, Yeo AR (1989) Effect of salinity on plant growth and crop yields. In: Cherry JH (ed) *Environmental stress in plants* Springer-Verlag, Berlin Heidelberg, pp101–119
- Howden R, Cobbett CS (1992) Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiol* 99: 100–107
- Iuchi S, Koyama H, Iuchi A et al (2007) Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. *Proc Natl Acad Sci U S A* 104:9900-9905
- Liu Q, Kasuga M, Sakuma Y et al (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10:1391–1406
- Murashige T, Skoog F, (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Neumann PM (1995) Inhibition of root growth by salinity stress: Toxicity or an adaptive biophysical response? In : Baluska F, Ciamporova M, Gasparikova O, Barlow

- PW (eds) Structure and Function of Roots. Developments in Plant and Soil Sciences. Kluwer Academic publishers, Dordrecht, Netherlands. pp 299-304.
- Orcutt DM, Nilsen ET (2000) Soil process and plant stress physiology. In: Orcutt DM, Nilsen ET (eds) Physiology of plants under stress: Soil and biotic factors, John Wiley & Sons, New York, pp177–235
- Sambatti BM, Caylor KK (2007) When is breeding for drought tolerance optimal if drought is random? *New Phytol* 175: 70–80
- Wu SJ, Ding L, Zhu JK (1996) SOS1, a genetic locus essential for salt tolerance and potassium acquisition. *Plant Cell* 8: 617–627
- Zhu JK (2001) Plant salt tolerance. *Trends Plant Sci* 6: 66–71

Table

Table 1. Comparison of *in vitro* assays and hydroponic culture to evaluate tolerance to osmotic stress in three lettuce cultivars. For experimental details, see legends to figures 2 and 3.

Cultivar	Growth		Survival	
	RDW ¹	RBR ²	SR ¹	RRR ²
Okayama-Saradana	31.0 ± 3.6	2.9 ± 2.1 ^b	45.8 ± 20.8	37.5 ± 6.9 ^b
Marino	34.8 ± 6.3	14.3 ± 9.0 ^{ab}	100.0 ± 0.0	100.0 ± 0.0 ^a
Blizzard	39.0 ± 4.4	35.7 ± 10.9 ^a	77.8 ± 22.2	97.6 ± 2.4 ^a

Data are the mean ± SE of at least three replications (n ≥ 5). Data within a column followed by different letters were significantly different at *p* = 0.01.

¹ Relative dry weight (RDW) and survival ratio (SR) in the hydroponic experiment were

calculated after 12 d drought stress as the relative percentage of the control plants.

² Root-bending ratio (RBR) and root recovery ratio (RRR) in the *in vitro* assays were calculated from the number of plantlets whose roots were bent on medium including 10% PEG, or that formed lateral roots in the rehydration treatment, respectively.

Legends to Figures

Figure 1. The root-bending and root recovery assays under osmotic stress. Five-day-old lettuce seedlings were transferred upside down from standard MS medium to the assay medium containing 200 mM NaCl for 7 d (A; the root-bending assay). The roots of the lefthand and central plantlets were bent, but the root of the righthand plantlet was not bent. The plantlets were subsequently transferred onto a medium lacking NaCl for the root recovery assay (B). Lateral roots were formed only in the lefthand plantlet after 14 d culture. Scale bars = 20 mm.

Figure 2. The effect of drought (A) and salinity (B) stress on growth and survival of the hydroponic-cultured lettuce cv. Okayama-Saradana seedlings. Twenty-five-day-old seedlings were drought stressed by halting watering (A). Thirty-day-old seedlings were treated with nutrient solution including a range of NaCl concentrations (B). Each plot represents the dry weight (○) or survival rate (●) relative to the control 0–14 d after the start of the drought stress and 16 d after imposing salinity stress. Vertical bars indicate the SE of at least three replications.

Figure 3. The root-bending assay and the root recovery assay of lettuce cv. Okayama-Saradana seedling responses to drought (A) and salinity stress (B).

Five-day-old seedlings were transferred onto assay media containing various concentrations of PEG (A) or NaCl (B) for 7 d. Subsequently, stressed plants were transferred to normal media for rehydration for another 14 d. The relative percentage of the controls were calculated from the number of plantlets whose roots were bent (○) or that formed lateral roots (●). Vertical bars indicate the SE of at least three replications. For the experimental details, see the legend to Figure 1.

Figure 1

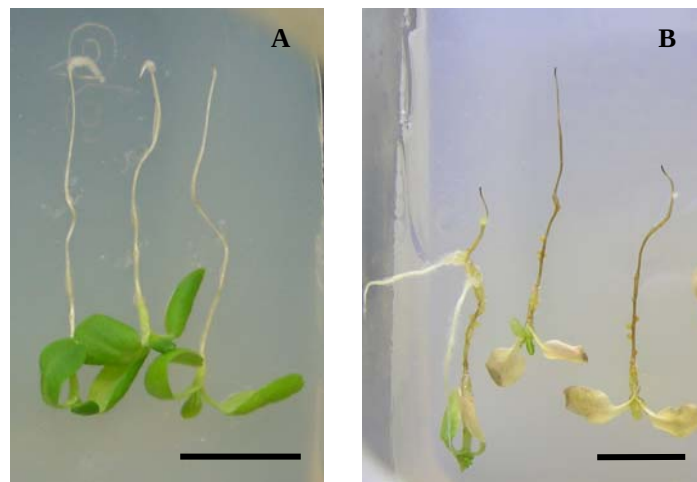


Figure 2

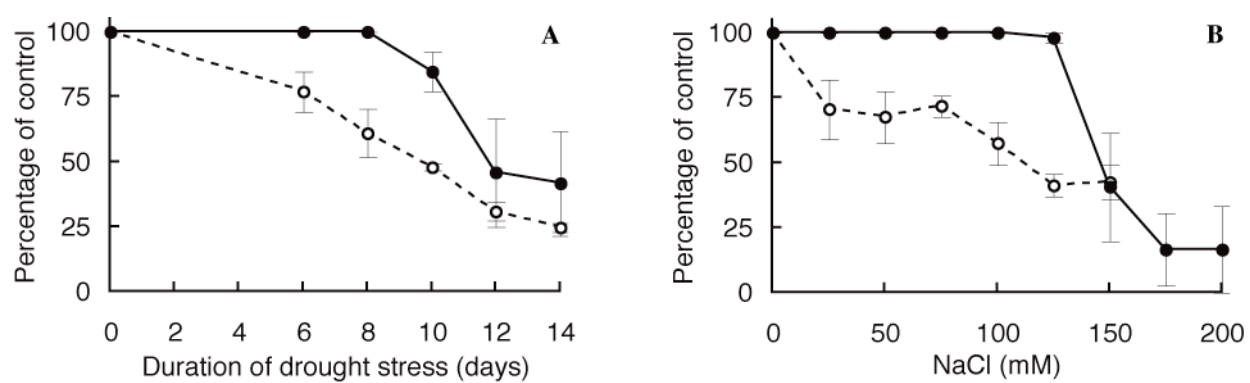


Figure 3

