Mixed cropping with ice plant alleviates the damage and the growth of cowpea under consecutive NaCl treatment and after the recovery from high salinity

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ABSTRACT

We investigated the alleviative effects of mixed cropping using ice plant, which is one of the salt-accumulating halophytes, on the damage and growth inhibition of cowpea, which is not tolerant to high salinity. Three cropping patterns (mono cropping of cowpea and ice plant and their combination) were tested. The plants were treated with 0, 100, 200 and 300 mM NaCl for 14 days (consecutive NaCl). The plants were also treated with NaCl for 3 days, followed by 2 weeks (short-term recovery) and 1 month (long-term recovery) recovery. Salinity levels for short-term recovery were similar to those of the consecutive experiment, while the concentration of long-term recovery was 250 mM. The alleviative effects of mixed cropping in the consecutive NaCl experiment were observed at 200 and 300 mM NaCl. Mixed cropping significantly reduced the Na content in the cowpea leaves at 200 and 300 mM NaCl compared with mono cropping. In addition, the Na content in the soil of mix-cropped cowpea at 200 and 300 mM NaCl was statistically lower than that of the mono cropping. Mixed cropping was effective to recover from high concentration of NaCl in the experiments of short- and long-term recovery. These results indicate that mixed cropping with a halophyte could be effective in mitigating the damage and growth inhibition of a glycophyte not only under salinity but also under recovery periods.

Soil salinity is one of the major problems leading to low agricultural productivity in the arid and semi-arid regions of the world. In Africa, more than 80 million ha of the agricultural land is affected by salinity (FAO and ITPS, 2015). In the dry countries of sub-Saharan Africa, for example, Namibia, soil salinity is one of the substantial concerns that cause low agricultural productivity. In this country, salt-affected soils cover about 5.13 million ha of land (Mashali, 1999) and arable land area is limited due to soil salinity. Salt-affected soils in Namibia are predominantly found in north central region, where is the highest population density (Coetzee, 2003; Mendelsohn et al., 2000). Thus, an agricultural system that could mitigate the adverse effects of salt stress on plant growth is needed.

One of the key factors to improve salt tolerance is reducing the amount of Na\textsuperscript{+} transported from roots to shoots (Munns and Tester, 2008), for which a few techniques have been proposed. The major technique would be identifying the genes related to Na\textsuperscript{+} transporters and introducing them into salt-sensitive plants for improving salt tolerance. Munns et al., (2012) reported that the gene \textit{TmHKT1;5-A} in the \textit{Nax2} locus encodes the Na\textsuperscript{+}-selective transporter located on the plasma membrane of root cells surrounding xylem vessels, and this transporter contributes to withdrawal of Na\textsuperscript{+} from xylem cells, thus reducing the amount of Na\textsuperscript{+} in the leaves of durum wheat. The durum wheat exhibited 25% higher yield than the near-isogenic line without the \textit{Nax2} locus in a field condition. Another unique technique that contributes to reduced Na\textsuperscript{+} in leaves is grafting (Albacete et al., 2009; Estañ et al., 2005). The authors reported using tomatoes that the concentration of Na\textsuperscript{+} is successfully reduced in the leaves of a salt-sensitive cultivar grafted onto a salt-tolerant cultivar, and the salt tolerance of the sensitive cultivar is improved. Phyto-amelioration, the method for the removal of excess salt from soils using salt-adapted plants, is also proposed (Ammari et al., 2008; Ravindran et al., 2007). The authors showed that fodder beet, one of the halophytes, is a potential candidate for rapid and efficient removal of salt from soils.
we showed that the growth and the physiological trait of flood-sensitive plants such as pearl millet and sorghum are ameliorated by mixed cropping with the flood-tolerant plant of rice, probably because of the transfer of oxygen from rice to pearl millet or sorghum (Awala et al., 2016; Iijima et al., 2016). These experiments and the phytoamelioration studies led us to hypothesise that the salt tolerance of salt-sensitive plants might be improved by mixed cropping with a halophyte because a halophyte would preferentially absorb and accumulate salt in the body, resulting in the reduction of salt contents in salt-sensitive plants. The root systems of a halophyte and a glycophyte should be tangled and closely interacted with each other to reduce salt contents in a glycophyte. Since there is neither space between two plants nor row space in mixed cropping, the cropping system would be appropriate to verify the hypothesis.

Cowpea (Vigna unguiculata) is the major crop in Africa, and more than three-fourth of the production area is spread in the region. Cowpea is a considerably adapted crop to drought and high temperature compared with other crops and has the yield potential of 1000 kg ha⁻¹ even though the annual rainfall is only 181 mm (Ehlers and Hall, 1997). Although cowpea has tolerance for various environmental stresses, salt stress strongly inhibits its growth primarily due to the accumulation of Na⁺ and Cl⁻ in the leaves (Cavalcanti et al., 2004; Gogile et al., 2013). Tavakkoli et al. (2010) reported that excess ion accumulation in faba bean leaves could be related to the reduction of chlorophyll content and photosynthetic rate, leading to plant growth inhibition. Thus, the reduction of excess ion in cowpea leaves is one of the candidate methods to mitigate the damage and the growth inhibition of cowpea under salinity.

The ice plant (Mesembryanthemum crystallinum) is a salt-accumulating halophyte originated in the Namib Desert on the western coast of southern Africa and is used as a leafy vegetable (Bohnert and Cushman, 2000). The ability of ice plant to accumulate Na⁺ in leaves and stems has been widely reported (Adams et al., 1998; Agarie et al., 2007; Hirokane et al., 2014), and excess Na⁺ is sequestered in specialised epidermal bladder cells (Adams et al., 1998; Agarie et al., 2007). Since ice plant also tolerates drought and high temperature (Abd El-Gawad and Shehata, 2014), the mixed cropping system using ice plant might reduce the ion content in the leaves of salt-sensitive tropical crops such as cowpea and mitigate the damage and growth inhibition.

The purpose of the present study is to investigate whether the salt tolerance of cowpea could be improved by mixed cropping with ice plant by reducing the salt concentration of cowpea leaves. We further discuss the usefulness of the mixed cropping system.

Materials and methods

Plant materials and salinity and recovery treatment

A series of experiments were conducted in the greenhouse of Kindai University in Nara prefecture (latitude 34°40′N, longitude 135°43′E). The average temperature and humidity during the experimental period (October 2015–May 2016) were maintained at 26 ± 3 °C and 65%, respectively. A gas heating system (KHF0255GF, Kusakabe, Japan) and automatic roof and window opening systems were used to maintain the temperature and humidity. The average photosynthetically active radiation (PAR) ranged from 386 to 947 μmol m⁻² s⁻¹. Metal halide lamps (BHF 200/220V500W, Iwasaki, Japan) were used to extend the day length to 14 h and maintain 350 μmol m⁻² s⁻¹ of PAR at the leaf canopy. Temperature, humidity and PAR were monitored using HOBO weather station (HOBO H21-001, USA).

Cowpea seeds (Vigna unguiculata, cv. Nakare) were obtained from the Seed Cooperative, Namibia, and the seeds of ice plant were purchased from Fukukaen Nursery & Bulb Co., Ltd, Japan. Before sowing, the seeds of cowpea and ice plant were surface sterilised with 2.5% (v/v) sodium hypochlorite for 5 min and rinsed with tap water for 20 min. The seeds of cowpea and ice plant were sown in plastic cell trays (50 long × 50 wide × 60 mm deep with an 8-mm-diameter hole at the bottom) filled with the growing medium (peat moss and ripe bark compost base, with coarse sand materials; Green plaza Yamacho original culture soil, Nara, Japan). The soil pH (H₂O) and EC were 5.39 and 0.85 ms m⁻¹, respectively. Total C and N in the soil were 177 and 4.1 g kg⁻¹, respectively. The mono- and mix-cropped ice plants were grown for 4 weeks prior to the sowing of cowpea to avoid the growth inhibition of ice plant by competition. The seeds of ice plant were directly sown in each cell (5–6 seeds/cell), and then the seedlings were thinned to one plant per cell at 5 days after emergence. The cell trays were placed in plastic trays (420 long × 290 wide × 60 mm deep) filled with tap water at a depth of 10 mm for 3 weeks. After growing for 3 weeks, the ice plant seedlings were transferred to water-circulating hydroponic boxes (660 × 660 × 200 mm, Home Hyponica 303, Kyowa, Japan) containing half the strength of Hoagland’s solution and grown for another week.

The seeds of cowpea were pre-germinated in a dark incubator (MIR-160, Sanyo Electric Biomedical, Japan) at 30 °C for 20 h. The pre-germinated cowpea seeds were sown in cell trays. The cowpea seeds mix-cropped with ice plant were relay-planted in the cell where the seedlings of 28-day-old ice plant were grown. The cell trays were placed in the hydroponic boxes containing the full-strength Hoagland’s solution for 14 days. The water level in the boxes was maintained at 10 mm depth and the solution was renewed once a week.

Materials and methods
After growing the cowpea plants for 2 weeks, salt stress was imposed to the mono- and mix-cropped plants. The cowpea plant was 14-day-old, and the second trifoliate leaves were fully expanded. The ice plant was 42-day-old, and the growth stage was late juvenile, which was judged according to Adams et al. (1998). Mono- and mix-cropped cowpea and ice plant were placed in plastic trays containing Hoagland’s solution with NaCl for 14 days (consecutive NaCl experiment). In this study, three different concentrations of NaCl (100, 200 and 300 mM) were applied to the plants, and control plants were grown in Hoagland’s solution without NaCl. The solution was filled in plastic trays at a depth of 20 mm and was continuously aerated by aeration pipes. The solution was renewed once a week. The treatments were arranged in a randomised complete block design with six replicates, and six plants were included in each replication.

The alleviative effects of mixed cropping on the inhibition of the physiological trait and the growth of cowpea during short- and long-term recovery were investigated. The plant materials were prepared as described above. In the short-term recovery experiment, the plants were treated with different concentrations of salinity (100, 200 and 300 mM) for 3 days according to the method mentioned above. After the treatment, the plants were grown with full-strength Hoagland’s solution without NaCl for 14 days. Control plants were grown without the NaCl treatment in both short- and long-term recovery experiments. In the long-term recovery experiment, the plants were treated with 250 mM NaCl for 3 days. After the treatment, the plants were transferred to 1/5000 a Wagner pot. In this study, sandy loam soil with a pH (H₂O) of 5.65, EC of 0.80 ms m⁻¹, total N of 0.03 g kg⁻¹ and total C of 0.46 g kg⁻¹ was used. The plants were grown with full-strength Hoagland’s solution without NaCl for 1 month. Then, the plants were watered every third day throughout the recovery period. The treatments were arranged in a randomised complete block design with three replicates, and six plants were included in each replication.

Measurements of physiological traits and growth parameters

The leaf greenness and the photosynthetic rate of the uppermost fully expanded trifoliate leaves of mono- and mix-cropped cowpea were measured using the SPAD metre (SPAD-502; Minolta Camera Co., Ltd, Japan) and a portable photosynthesis analyser (LCpro SD, ADC BioScientific, UK), respectively. For the measurement of photosynthetic rates, the photosynthetic photon flux density and the concentration of CO₂ in a chamber were set to 1300 μmol photon m⁻² s⁻¹ and 380 μmol mol⁻¹ s⁻¹, respectively. Temperature in the chamber was maintained at the range of 26–28 °C. Three moderately grown plants were used for the measurement per replication. The physiological traits were monitored after 3, 6, 9 and 12 days of salt treatment in the experiments of consecutive NaCl and short-term recovery. In the long-term recovery experiment, SPAD values and photosynthetic rates were monitored for 1 month at 5-day intervals. During the monitoring, new leaves had emerged and developed in the mono- and mix-cropped control cowpea plants and in the salt-treated mix-cropped cowpea plants during the recovery. The new leaves were replaced by the uppermost fully expanded leaves from 20 days after the recovery. Therefore, we used the new leaves for the measurement of SPAD values and photosynthetic rate. In the salt-treated cowpea plants of mono cropping, the uppermost fully expanded leaves started to wilt after the measurement of physiological traits at 10 days of recovery. However, new leaves had emerged and developed during the recovery, and the new leaves were replaced by the uppermost fully expanded leaves from 15 days of recovery. Therefore, we used the new leaves for the measurement of SPAD values and photosynthetic rate.

At the end of each experiment, the shoots of cowpea and ice plant were harvested, and then they were oven-dried at 70 °C for 72 h for the measurement of shoot dry weight. Shoot relative growth rate (RGR) was calculated using the following equation according to Hoffmann and Poorter (2002):

\[
RGR = \frac{(\ln W_2 - \ln W_1)}{(t_2 - t_1)}
\]

where \(\ln W_2\) and \(\ln W_1\) are the means of the natural logarithm-transformed final (at the end of salinity stress) and initial (just before salinity stress) shoot dry weights, respectively, and \(t_2-t_1\) indicates the duration of the salinity stress period. A competitive ratio (CR) was calculated using the shoot biomass production of the two component crops following the method of Willey and Rao (1980) as follows:

\[
CR(C_p) = \frac{(Y_{C_p-mix}/Y_{C_p-mono})}{(Y_{I_p-mix}/Y_{I_p-mono})}
\]

\[
CR(I_p) = \frac{(Y_{I_p-mix}/Y_{I_p-mono})}{(Y_{C_p-mix}/Y_{C_p-mono})}
\]

where \(Y\) is the shoot dry weight of the plant.

After the measurement of dry weight, the uppermost fully expanded leaves were used for the analysis of Na⁺ and K⁺ content in the consecutive NaCl experiment. The leaves were grinded with a pestle and a mortar, and 50 mg of the leaves was used for subsequent measurements. The Na⁺ and K⁺ ions were extracted according to Mitsuya et al. (2002). In brief, the leaves were incubated in 10 ml of 1 M HCl solution at 90 °C for 2 h and then incubated using a rotor (EYELA MMS-3010, Tokyo Rikakikai Co., Ltd, Japan).
Results

Alleviative effects of mixed cropping under consecutive NaCl treatment

The time course measurement of SPAD values was conducted to investigate the alleviative effects of mixed cropping on the damage of cowpea leaves (Figure 1), because the decrease in chlorophyll content is one of the typical symptoms under salinity (Yamane et al., 2004). The SPAD values of mono and mixed cropping under control were around 50 during the experiment (Figure 1(A)). The SPAD values of mono and mixed cropping under 100 mM NaCl were maintained around 50 (Figure 1(B)), suggesting that cowpea could tolerate salinity up to 100 mM NaCl, regardless of the cropping patterns. The SPAD values of the mono-cropped cowpea treated with 200 and 300 mM NaCl rapidly decreased after the treatment (Figure 1(C) and (D)). The values after the treatment with 200 and 300 mM NaCl for 12 days were 17.2 (Figure 1(C)) and 0 (Figure 1(D)), respectively. Mixed cropping using ice plant was effective in mitigating the decrease in SPAD values caused by the high concentration of NaCl (Figure 1(C) and (D)). The SPAD value of mix-cropped

for 24 h at room temperature. The solutions were diluted and filtered, and an atomic absorption spectrophotometer (Hitachi, Z-2300, Japan) was used to measure the concentration of Na⁺ and K⁺. The same procedure was used to measure the Na⁺ concentration in the soil of mono and mixed cropping. The soil samples were air-dried and then passed through a 2 mm sieve to remove plant roots. For the extraction of Na ions, 50 mg of the soil was used. The methods of the extraction and the measurement were same to the leaf ion measurement.

Statistical analysis

The statistical significance between mono and mixed cropping in Figures 1–8 was tested using Student’s t-test with the level of statistical significance taken as $p < 0.05$ and $p < 0.01$. In the data of Table 1, Figures 4 and 9, one-way analysis of variance (ANOVA) was first applied for statistical evaluation. If an ANOVA was significant, post hoc analyses were conducted using Tukey–Kramer multiple comparison test, with the level of statistical significance taken as $p < 0.01$. The Excel software for Windows 2012 (SSRI Japan, Co. Ltd) was used for the statistical analysis.
cowpea treated with 200 and 300 mM NaCl was statistically higher than that of the mono-cropped plant at each measurement day.

The time course measurement of photosynthetic rates was conducted to explore the alleviative effects of mixed cropping on the leaf physiological activities of cowpea. The photosynthetic rates of mono and mixed cropping under control during the experiment were >15.0 μmol m⁻² s⁻¹, and the cropping pattern did not influence the photosynthetic rates (Figure 2(A)). The photosynthetic rates of mono and mixed cropping after 3 days of treatment with 100 mM NaCl were lower than the control, and mixed cropping was not effective in mitigating the decrease in the photosynthetic rate (Figure 2(B)). Figure 2(C) and (D) shows the photosynthetic rates of mono- and mix-cropped cowpea treated with 200 and 300 mM NaCl. The photosynthetic rates of both cropping patterns were greatly suppressed by the treatment compared with the control. The rate of mono-cropped cowpea treated with 300 mM NaCl was almost 0 at 6 days after the treatment. However, the suppression of photosynthesis observed in mono cropping after the treatment with 200 and 300 mM NaCl was effectively mitigated by mixed cropping with ice plant. In particular, the photosynthetic rates of mixed cropping at 3, 6 and 9 days after the treatment with 300 mM NaCl were statistically higher than those of mono cropping, while the value at day 12 was 0.

Figure 3 shows the changes in Na⁺ and K⁺ content in the leaves of cowpea and ice plant of control and after treatments with 100, 200 and 300 mM NaCl for 14 days. The treatments with 100, 200 and 300 mM NaCl reduced the K⁺ content in the leaves of cowpea (Figure 3(A)) and ice plant (Figure 3(B)) compared with that of the control. The K⁺ content of mix-cropped cowpea under 100, 200 and 300 mM NaCl was similar to that of mono-cropped plants (Figure 3(A)). On the other hand, the K⁺ content of mix-cropped ice plant at 100, 200 and 300 mM NaCl was slightly higher than that of mono-cropped plants, though the difference was not statistically significant (Figure 3(B)). The Na⁺ content in the leaves of cowpea and ice plant of control treated with salt stresses increased with increasing NaCl concentration supplied to the plants (Figure 3(C) and (D)). The Na⁺ content of cowpea and ice plant of mixed cropping under control and 100 mM NaCl was slightly higher than that of mono cropping. On the other hand, the Na⁺ content of cowpea and ice plant of mixed cropping at 200 and 300 mM NaCl

Figure 2. Time course of the photosynthetic rate of cowpea at 0 (A), 100 (B), 200 (C) and 300 (D) mM NaCl in the experiment of consecutive NaCl treatment. Notes: The data are means ± SE (n = 6). The symbols of * and ** indicate the significant difference between mono and mix cropping in each day at p < 0.05 and p < 0.01 by Student’s t-test, respectively.
other cropping patterns. However, the soil Na$^{+}$ content of mix-cropped cowpea and ice plant treated with 200 and 300 mM NaCl was statistically lower than that of mono-cropped cowpea.

Figure 5(A) and (B) shows shoot dry weights of cowpea and ice plant of mono and mixed cropping after the treatment with or without NaCl for 14 days. The shoot dry weights of cowpea mix-cropped with ice plant under control and 100 mM NaCl were statistically lower than those of mono cropping. However, the shoot dry weights of mix-cropped cowpea at 200 and 300 mM NaCl were slightly higher than those of mono cropping. The shoot dry weights of ice plant in mixed cropping at all concentrations of NaCl were higher than those of mono cropping, and a statistical difference was observed under control and

Figure 3. K$^{+}$ (A, B) and Na$^{+}$ (C, D) content and Na$^{+}$/K$^{+}$ ratio (E, F) of the leaves in cowpea and ice plant at 14 days after treatment with 100, 200 and 300 mM NaCl in the experiment of consecutive NaCl treatment.

Notes: The data are means ± SE (n = 6). The symbols of * and ** indicate the significant difference between mono and mix cropping in each NaCl concentration at $p < 0.05$ and $p < 0.01$ by Student's t-test, respectively.

were lower than that of mono cropping, and a statistical difference was evident for cowpea (Figure 3(C)). The lower Na$^{+}$ content of cowpea exhibited a statistical decrease in Na/K ratio at 200 and 300 mM NaCl (Figure 3(E)). In the ice plant, the Na/K ratio of mixed cropping at 100, 200 and 300 mM NaCl was lower than that of mono cropping (Figure 3(F)), which is induced by the higher K$^{+}$ content in each treatment.

Figure 4 shows the soil Na$^{+}$ content of mono and mixed cropping after the treatment with or without NaCl for 14 days. The soil Na$^{+}$ content increased with increasing salinity stress in all cropping patterns. In the soil treated with 100 mM NaCl, the Na$^{+}$ content was not statistically significant among the cropping systems, though Na$^{+}$ content of mono-cropped cowpea tended to be higher than other cropping patterns. However, the soil Na$^{+}$ content of mix-cropped cowpea and ice plant treated with 200 and 300 mM NaCl was statistically lower than that of mono-cropped cowpea.

Figure 5(A) and (B) shows shoot dry weights of cowpea and ice plant of mono and mixed cropping after the treatment with or without NaCl for 14 days. The shoot dry weights of cowpea mix-cropped with ice plant under control and 100 mM NaCl were statistically lower than those of mono cropping. However, the shoot dry weights of mix-cropped cowpea at 200 and 300 mM NaCl were slightly higher than those of mono cropping. The shoot dry weights of ice plant in mixed cropping at all concentrations of NaCl were higher than those of mono cropping, and a statistical difference was observed under control and
100 mM NaCl. Figure 5(C) and (D) shows the RGR of cowpea and ice plant of mono and mixed cropping during 14 days under control and NaCl treatments. The trend of RGR of cowpea was similar to that of shoot dry weight, though the difference between mono and mixed cropping was not statistically different. The RGR values of ice plant mixed-cropped with cowpea were higher than those of mono cropping, except for RGR at 200 mM NaCl.

Table 1 shows the RY, RYT and CR of cowpea and ice plant of control and after treatments with 100, 200 and 300 mM NaCl, which were calculated using shoot dry weight. The RY values of cowpea under control and 100 mM NaCl were lower than those of ice plant because of the competition. However, the competition was suppressed at 200 and 300 mM NaCl. The RY values of cowpea and ice plant at 200 and 300 mM NaCl were higher than 1.0. In addition, the CR values of both plants at 200 and 300 mM NaCl were around 1.0. The RYT values were always more than 2.0, indicating the successful combination in terms of mixed cropping.

Figure 4. Soil Na⁺ content under mono and mix-cropping at 14 days after treatment with 100, 200 and 300 mM NaCl in the experiment of consecutive NaCl treatment. Notes: The data are means ± SE (n = 6). Different letters above the bars indicate significant differences at $p < 0.01$ by Tukey–Kramer multiple comparison test.

Figure 5. Shoot dry weight (A, B) and relative growth rate (RGR) (C, D) of cowpea and ice plant of mono and mixed cropping at 14 days after treatment with 100, 200 and 300 mM NaCl in the experiment of consecutive NaCl treatment. Notes: The data are means ± SE (n = 6). The symbols of * and ** indicate the significant difference between mono and mix cropping in each NaCl treatment at $p < 0.05$ and $p < 0.01$ by Student’s t-test, respectively.
Alleviative effects of mixed cropping after short-term recovery from different concentrations of salt stress

Figure 6 shows the time course of the SPAD values of cowpea of mono and mixed cropping after recovery from the treatments with 100, 200 and 300 mM NaCl for 3 days. The SPAD values of mono and mixed cropping in the control were similar at each recovery period (Figure 6(A)). In addition, the SPAD values of cowpea of mono and mixed cropping after the recovery from 100 (Figure 6(B)) and 200 mM NaCl (Figure 6(C)) were similar in both the cropping patterns. However, the SPAD values of monocropped cowpea after recovery from 300 mM NaCl rapidly decreased and the value reached almost 0 after 12 days of recovery (Figure 6(D)). Although the SPAD value of cowpea mix-cropped with ice plant gradually decreased, the value was always statistically higher than that of mono-cropped plants at each recovery period.

Figure 7 shows the time course of the photosynthetic rates of cowpea after recovery from the treatments with 100, 200 and 300 mM NaCl for 3 days. The photosynthetic rates of mixed cropping of control were slightly lower than those of mono cropping, though the differences were not statistically significant (Figure 7(A)). The treatments with 100, 200 and 300 mM NaCl for 3 days reduced the photosynthetic rates of mono and mixed cropping. The photosynthetic rates of mono- and mix-cropped cowpea treated with 100 (Figure 7(B)) and 200 mM NaCl (Figure 7(C)) gradually recovered during the recovery period and the trend of mix-cropped cowpea was similar to mono cropping. However, the photosynthetic rates of mono-cropped cowpea treated with 300 mM NaCl did not recover (Figure 7(D)). On the other hand, the photosynthetic rates of mix-cropped cowpea gradually recovered, and statistical differences between mono and mixed cropping were observed after 3, 9 and 12 days of recovery (Figure 7(D)).

Figure 8 shows the shoot dry weights and RGR after 14 days of recovery from the treatments with 100, 200 and 300 mM NaCl for 3 days. The shoot dry weights of monocropped cowpea of control and after the recovery from 100 mM NaCl were lower than those of mono cropping, because of the competition with ice plant (Figure 8(A)). The difference observed after the recovery from 100 mM NaCl was statistically significant (Figure 8(A)). However,
Figure 9(A) shows the time course of the SPAD values of mono- and mix-cropped cowpea control and recovery from 250 mM NaCl. The SPAD values of mono- (white circle) and mix- (white triangle) cropped cowpea under control (open marks) were not statistically different, while a significant difference was observed only at day 15 after the recovery. The SPAD values of mono-cropped cowpea after recovery from 250 mM NaCl (grey circle) rapidly decreased from day 0 to 10. Although the new uppermost fully expanded leaves were replaced from day 15 of recovery, the SPAD values were statistically lower than those of other treatments at each recovery day. In the salt-treated cowpea mix-cropped with ice plant (grey triangle), the SPAD values of the uppermost fully expanded leaves were statistically lower than those of mono-cropped control from day 0 to 15. However, the SPAD values of the leaves were maintained at similar level from day 0 to 15, and the leaves did not wilt. The new uppermost expanded leaves were replaced from day 20 of recovery, and the SPAD values were slightly lower than those of the control of mono- and mix-cropped cowpea, although no significant differences were observed.

Figure 7. Time course of the photosynthetic rate of mono and mix cropped cowpea in the short-term recovery experiment. Cowpea plants were treated with 100 (B), 200 (C) and 300 (D) mM NaCl for three days, and then they were grown under the nutrient solution without NaCl. Control plants (A) were grown without NaCl treatment.

Notes: The data were taken after 0, 3, 6 and 9 days of recovery. Each value is the mean ± SE (n = 3). The symbols of * and ** indicate the significant difference between mono and mixed cropping in each day at p < 0.05 and p < 0.01 by Student’s t-test, respectively.

An opposite trend was observed in cowpea after recovery from 200 to 300 mM NaCl. The shoot dry weights of mix-cropped cowpea after recovery from 200 to 300 mM NaCl were higher than those of mono cropping, and the difference observed with 300 mM NaCl was statistically significant (Figure 8(A)). The shoot dry weights of mix-cropped ice plant in all treatments were higher than those of mono cropping after the recovery, while the differences were not statistically significant (Figure 8(B)). The RGR values of cowpea and ice plant showed a similar trend as observed in the shoot dry weight (Figure 8(C) and (D)). Statistically significant differences between mono and mixed cropping were not observed in all treatments.

Alleviative effects of mixed cropping after long-term recovery from high concentration of salt stress

In the short-term recovery experiment, mixed cropping with ice plant was more effective on the recovery of physiological traits and the growth of cowpea after the high concentration of salt stress (300 mM NaCl). Therefore, we evaluated the alleviative effect for longer period (1 month). Figure 9(A) shows the time course of the SPAD values of mono- and mix-cropped cowpea control and recovery from 250 mM NaCl. The SPAD values of mono- (white circle) and mix- (white triangle) cropped cowpea under control (open marks) were not statistically different, while a significant difference was observed only at day 15 after the recovery. The SPAD values of mono-cropped cowpea after recovery from 250 mM NaCl (grey circle) rapidly decreased from day 0 to 10. Although the new uppermost fully expanded leaves were replaced from day 15 of recovery, the SPAD values were statistically lower than those of other treatments at each recovery day. In the salt-treated cowpea mix-cropped with ice plant (grey triangle), the SPAD values of the uppermost fully expanded leaves were statistically lower than those of mono-cropped control from day 0 to 15. However, the SPAD values of the leaves were maintained at similar level from day 0 to 15, and the leaves did not wilt. The new uppermost expanded leaves were replaced from day 20 of recovery, and the SPAD values were slightly lower than those of the control of mono- and mix-cropped cowpea, although no significant differences were observed.
The photosynthetic rates of mix-cropped cowpea should have been induced by competition with the ice plant. However, the reduction was greater than that observed in the experiments of consecutive NaCl treatment (Figure 2(A)) and short-term recovery (Figure 7(A)). One of the candidate factors induced the great reduction could be transplanting shock. Since the transplanting shock was observed only in mix-cropped cowpea (Figure 9(A), open triangle) but not mono-cropped cowpea (Figure 9(A), open circle), mix cropping could enhance the transplanting shock. However, the photosynthetic rates of mix-cropped cowpea gradually increased and the value reached similar level to the mono cropping after day 15 of recovery. The treatment with 250 mM NaCl for 3 days suppressed the photosynthetic rates of cowpea of mono and mixed cropping. In the mono-cropped cowpea (grey circle), the photosynthetic rates slightly increased during the recovery, while the leaves wilted after the measurement at 10 days of recovery. Although the new uppermost fully expanded leaves were replaced, the photosynthetic rates were statistically lower than those of other treatments after 15 days of recovery. On
recovery, the photosynthetic rate of the new uppermost fully expanded leaves was similar to that of the control of mono and mixed cropping.

the other hand, the photosynthetic rates of mix-cropped cowpea treated with 250 mM NaCl for 3 days (grey triangle) rapidly increased after the recovery. After 20 days of recovery, the photosynthetic rate of the new uppermost fully expanded leaves was similar to that of the control of mono and mixed cropping.
After 1 month of recovery, the shoots of cowpea and ice plant were sampled and shoot dry weight and RGR were evaluated (Figure 10). Figure 10(A) shows the shoot dry weight of mono- and mix-cropped cowpea. The shoot dry weight of mix-cropped cowpea after the treatment with NaCl was statistically lower than that of cowpea treated with NaCl treatment. Figure 10(C) shows RGR of mono- and mix-cropped cowpea. The RGR value of mix-cropped cowpea of control was slightly higher than that of mono cropping. The RGR value of mono-cropped cowpea after recovery from 250 mM NaCl was almost 0. The shoot dry weight of mix-cropped cowpea after the recovery was statistically higher than that of mono cropping. Figure 10(B) shows the shoot dry weights of ice plant of mono and mixed cropping. The treatment with NaCl promoted the growth of ice plant during the recovery period. In the control plant, the dry weight of mixed cropping was slightly higher than that of mono cropping. However, the shoot dry weight of mix-cropped cowpea treated with salt stress was slightly lower than that of mono cropping. Figure 10(D) shows RGR of ice plant of mono and mixed cropping. In both control and salt-treated plants, the RGR value of mixed cropping was higher than that of mono cropping.

Discussion

Mixed cropping is effective in mitigating the damage and growth inhibition of cowpea under consecutive NaCl treatment

Cowpea could have the ability to survive under moderate salinity conditions, because the chlorophyll content measured by the SPAD metre, which is one of the indicators of oxidative damage under salinity (Yamane et al., 2004), did not decrease during the consecutive treatment with 100 mM NaCl compared with that of mono cropping (Figure 1(B)). Mitsuya et al. (2002) suggested that leaf damage under salinity is induced after the amount of Na+ in leaves exceeds a threshold value. In the present study, the Na+ contents in the leaves of mono- and mix-cropped cowpea at 100 mM NaCl were 55 and 59 mg g DW⁻¹, respectively, and the values were about 2.5-fold higher than those in the cowpea leaves of control (Figure 3(C)). Thus, the Na+ content was within the threshold value of cowpea, and cowpea was able to suppress the Na⁺ content in the leaves below a toxic level at 100 mM NaCl. On the other hand, the SPAD values of mono- and mix-cropped cowpea treated with 200 and 300 mM NaCl (Figure 1(C) and (D)) were lower than those of the control (Figure 1(A)) or at 100 mM NaCl (Figure 1(B)), suggesting that the Na⁺ content in the cowpea leaves exceeded above the threshold value by the treatment with high concentrations of NaCl. However, the reduction of SPAD value observed in cowpea mix-cropped with ice plant was significantly alleviated compared with mono cropping (Figure 1(C) and (D)). In addition, the photosynthetic rates of cowpea mix-cropped with ice plant at 200 and 300 mM NaCl were statistically higher than those of mono cropping (Figure 2(C) and (D)). The Na⁺ contents in the cowpea leaves mix-cropped with ice plant at 200 and 300 mM NaCl significantly decreased compared with those in mono cropping (Figure 3(C)), resulting in the lower Na/K ratio (Figure 3(E)). Although there is strong correlations between increases in leaf Na concentrations, resulting in high Na/K ratio, and the reduction in photosynthesis, the detail mechanisms have not been fully elucidated (Munns et al., 2006). In salt-tolerant barley, which can maintain photosynthesis under salt stress, the favourable Na/K ratio in the cytoplasm is preserved at high leaf Na concentrations (200–300 mM) (Munns et al., 2006), probably because of compartmentalisation of Na⁺ into vacuoles. The high concentration of Na⁺ in tomato leaves, resulting in high Na/K ratio, inhibits the photosynthetic enzyme activities such as Rubisco, chloroplastic fructose-1,6-bisphosphatase, fructose-1,6-bisphosphate aldolase, and phosphoribulokinase (Yang et al., 2008). Thus, the maintenance of photosynthetic capacity could be achieved by the maintenance of high K and low Na, resulting in low Na/K ratio in the cytoplasm of mesophyll cells (Munns et al., 2006). In addition, the soil Na⁺ content in mixed-cropping cowpea treated with 200 and 300 mM NaCl was significantly lower than that of mono-cropped cowpea (Figure 4). These results indicate that mixed cropping with a halophyte is effective in the decrease in ionic effects of salinity due to the reduction of the Na⁺ content absorbed by the glycophyte, leading to the mitigation of the damage and maintenance of photosynthesis of glycophyte. The decrease in chlorophyll content observed under salinity is a typical symptom of oxidative damage in the chloroplast (Yamane et al., 2004). Thus, mixed cropping may alleviate the salt-induced oxidative stress by decreasing the Na⁺ content in cowpea leaves.

The mixed cropping system tested would be also effective in the decrease in osmotic effects of salinity, judging from the data of the photosynthetic rate and Na⁺ contents in leaves and soils, as shown in Figures 2–4, as a whole. Cavalcanti et al. (2004) observed a rapid decrease in the transpiration rate of cowpea after the treatment with 200 mM NaCl. The authors suggested that the growth reduction of cowpea induced by 200 mM NaCl could be due to the decrease in transpiration and photosynthetic rates primarily caused by the osmotic component of salinity. The decreased rate of shoot growth under salinity...
could be primarily due to the osmotic effects, because the osmotic effect induces stomatal closure, followed by the reduction of photosynthetic rate (Munns et al., 2006).

In the present study, the shoot dry weight and RGR values of mix-cropped cowpea were slightly higher than those of mono cropping (Figure 5(A) and (C)) at both 200 and 300 mM NaCl, probably because of the higher photosynthetic rates (Figure 2). The leaf (Figure 3(C)) and soil (Figure 4) Na⁺ contents in mix-cropped cowpea at 200 and 300 mM NaCl were statistically lower than those of mono-cropped cowpea. These results suggest that mixed cropping with ice plant ameliorated the inhibition in both root environment and shoot growth of cowpea by decreasing the adverse effects of the osmotic component of salinity due to the reduction of leaf and soil Na⁺ contents.

**Alleviative effect of mixed cropping on the damage and growth of cowpea after recovery from high concentration of NaCl treatment**

In a previous study using cowpea, the emergence and growth of new leaves were found to be important for the restoration of regrowth after recovery periods (Silveira et al., 2001). In addition to the growth of new leaves, the results of the present study suggest that maintenance of the photosynthetic rate in the uppermost fully expanded leaves of cowpea during recovery periods is also important for the restoration of regrowth. In the long-term recovery experiment, the new leaves of the mono-cropped cowpea treated with 250 mM NaCl emerged and grew during the recovery period, and the new leaves were replaced by the uppermost fully expanded leaves from 15 days of recovery. However, the physiological activity of the new leaves was lower than that of other treatments (Figure 9(B)). On the other hand, the photosynthetic rate of the new leaves of mix-cropped cowpea from day 20 to 30 of recovery was similar to that of the control (Figure 9(B)). In the mono-cropped cowpea treated with 250 mM NaCl, the uppermost fully expanded leaves wilted during the recovery. However, the uppermost fully expanded leaves in mix-cropped cowpea treated with 250 mM NaCl did not wilt and the photosynthetic rate rapidly recovered from day 0 to 15 of recovery (Figure 9(B)). The shoot dry weight (Figure 10(A)) and RGR (Figure 10(C)) values of mixed cropping after recovery from 250 mM NaCl were statistically higher than those of mono cropping. These results suggest that maintenance of the photosynthetic rate in the uppermost fully expanded leaves during recovery period is important for the development of new leaves and the subsequent regrowth of cowpea during a recovery period. Mixed cropping with ice plant is useful to restore the regrowth by the suppression of the salt-induced damage in uppermost fully expanded leaves.

**The application of mixed cropping to a field**

Methods to mitigate the adverse effects of salt stress on crop production need to be urgently developed, because of the progressive salinisation of productive agricultural lands. Mixed cropping has not been tested as a method to mitigate the adverse effects of salinity. In the present study, we demonstrated for the first time that mixed cropping with ice plant, a salt-accumulating halophyte, could alleviate the salinity stress of cowpea. The mixed cropping system using ice plant has not been applied, probably because ice plant has not been a major crop. However, ice plant is utilised as a raw or cooked vegetable (Abd El-Gawad and Shehata, 2014; Herppich et al., 2008) and is principally used for medicinal purposes (Abd El-Gawad and Shehata, 2014; Deters et al., 2012; Ibtissem et al., 2012). Thus, ice plant could be synergised in a mixed cropping system. Since cowpea improves the fertility of poor nutrient soils by fixing nitrogen, it is often used in mixed cropping systems with cereals such as maize, millet and sorghum in farming systems in sub-Saharan Africa (Dugje et al., 2009; Matusso et al., 2014). The growth of ice plant mix-cropped with cowpea was slightly better than that of mono cropping, while the shoot dry weight of ice plant mix-cropped with cowpea after recovery from 250 mM NaCl was lower than that of mono cropping (Figure 10(B)). The growth enhancement of ice plant by mix cropping with cowpea may be due to the improvement of nitrogen fertility in soils by fixing nitrogen. In addition, the growth of cowpea during the treatment and recovery from the high concentration of NaCl was mitigated. Thus, the mixed cropping system tested in the present study could improve the growth of both cowpea and ice plant simultaneously. These results suggest that farmers can grow both crops under saline soils and can use both crops for their consumption or as cash crops, which will improve their livelihoods. However, the growth of cowpea under control and moderate salinity (100 mM NaCl) was suppressed (Table 1). Since cowpea mix-cropped with ice plant was relay-planted in a cell tray where the seedlings of 28-day-old ice plant were grown, the growth suppression of cowpea could be induced by the high competition for resources such as light, water and nutrient. Thus, further studies are needed to reduce the competition, and the cropping system which can alleviate the adverse effects under moderate levels of salt stress should be developed.

The conditions for salinity treatment used in the present study, that is, 3–12 days exposure to salt stress followed by recovery period of two weeks or 1 month on hydroponically grown plants in cell trays, were designed to model the sea water flash flooding cases seen in many fields near the sea shore. However, most of the phenomenon seen in the semi-arid region where salinity stress persists for prolonged period and hardly removed in such a short
period of time should be far different from the cases of sea water flooding. We further need to test the effectiveness of this cropping system in salt tolerance under the prolonged period of salinity stresses without any recovery. In addition, the root system of the mixed crops in real field condition should be much spread, although some outgrowth of root from the 8 mm holes at the bottom of cell trays would be expected. The experimental set up used in this study is just a simplified model system to evaluate the possibility of the closed mixed-cropping technique. Field evaluation considering these should be conducted further to clarify the potentiality to be used in the practical situation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Abd El-Gawad, A. M., & Shehata, H. S. (2014). Ecology and development of Mesembryanthemum crystallinum L. in the deltaic Mediterranean coast of Egypt. *Egyptian Journal of Basic and Applied Sciences*, 1, 29–37. doi:10.1016/j.ejbas.2014.02.003

Adams, P., Nelson, D., Yamada, S., Jensen, W. C. R. G., Bohnert, H. J., & Griffiths, H. (1998). Growth and development of Mesembryanthemum crystallinum (Aizoaceae). *New Phytologist*, 138, 171–190. doi:10.1046/j.1469-8137.1998.00111.x

Agarie, S., Shimoda, T., Shimizu, Y., Baumann, K., Sunagawa, H., Kondo, A., ... Cushman, J. C. (2007). Salt tolerance, salt accumulation, and ionic homeostasis in an epidermal bladder-cell-less mutant of the common ice plant *Mesembryanthemum crystallinum*. *Experimental Botany*, 58, 1957–2967. doi:10.1093/jxb/erm057

Albacete, A., Martínez-Andújar, C., Ghanem, M. E., Acosta, M., Sánchez-Bravo, J., Asins, M. J., ... Pérez-Alfocea, F. (2009). Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato. *Plant, Cell and Environment*, 32, 928–938. doi:10.1111/j.1365-3040.2009.01973.x

Ammari, T. G., Tahboub, A. B., Saoub, H. M., Hattar, B. I., & Al-Zu’bi, Y. A. (2008). Salt removal efficiency as influenced by phyto-amelioration of salt-affected soils. *Journal of Food, Agriculture and Environment*, 6, 456–460.

Awala, S. K., Yamane, K., Izumi, Y., Fujioka, Y., Watanabe, Y., Wada, K. C., ... Iijima, M. (2016). Field evaluation of mixed-seeding with rice to alleviate flood stress for semi-arid cereals. *European Journal of Agronomy*, 80, 105–112. doi:10.1016/j.eja.2016.07.003

Bohnert, H. J., & Cushman, J. C. (2000). The ice plant cometh: Lessons in abiotic stress tolerance. *Journal of Plant Growth Regulation*, 19, 334–346. doi:10.1007/s003440000033

Cavalcanti, F. R., Abreu, J. T., Martins-Miranda, A. S., Viégas, R. A., & Silveira, J. A. G. (2004). Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. *New Phytologist*, 163, 563–571. doi:10.1111/j.1469-8137.2004.01139.x

Coetzee, M. E. (2003). *Characteristics of Namibian soils in a nutshell*. Spotlight on agriculture 65. Windhoek: MAWRD.

Deters, A. M., Meyer, U., & Stintzing, F. C. (2012). Time-dependent bioactivity of preparations for cactus pear (*Opuntia ficus indica*) and ice plant (*Mesembryanthemum crystallinum*) on human skin fibroblasts and keratinocytes. *Journal of Ethnopharmacology*, 142, 438–444. doi:10.1016/j.jep.2012.05.014

Dugje, I. Y., Omoigui, L. O., Ekeleme, F., Kamara, A. Y., & Ajeigbe, H. (2009). *Farmers’ guide to cowpea production in West Africa* (p. 1). Ibadan: IITA.

Ehlers, J. D., & Hall, A. E. (1997). Cowpea (*Vigna unguiculata* L. Walp.). *Field Crops Research*, 53, 187–204. doi:10.1016/S0378-4290(97)00031-2

Estañ, M. T., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Flowers, T. J., & Bolain, M. C. (2005). Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. *Journal of Experimental Botany*, 56, 703–712. doi:10.1093/jexpbot/eri027

FAO and ITPS. (2015). *Status of the World's Soil Resources (SWSR) – Main Report*. Rome, Italy: Food and Agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils. pp. 252.

Gogile, A., Andargie, M., & Muthuswamy, M. (2013). Screening selected genotypes of cowpea (*Vigna unguiculata* (L.) Walp.) for salt tolerance during seedling growth stage. *Pakistan Journal of Biological Sciences*, 16, 671–679. doi:10.3923/pjbs.2013.671.679

Herppich, W. B., Huyskens-Keil, W., & Huyskens-Keil, S. (2008). Effects of saline irrigation on growth, physiology and quality of Mesembryanthemum crystallinum L., a rare vegetable crop. *Journal of Applied Botany and Food Quality*, 82, 47–54.

Hirokane, T., Ooba, M., Shimada, S., & Toyoda, H. (2014). Effects of water and salinity stress on growth of the ice plant. *Journal of Arid Land Studies*, 24, 161–164.

Hoffmann, W. A., & Poorter, H. (2002). Avoiding bias in calculations of relative growth rate. *Annals of Botany*, 90, 37–42. doi:10.1093/aob/mcf140

Ibtissem, B., Abdelly, C., & Sfar, S. (2012). Antioxidant and antibacterial properties of *Mesembryanthemum crystallinum* and *Carpobrotus edulis* extracts. *Advances in Chemical Engineering and Science*, 2, 359–365. doi:10.4236/aces.2012.23042

Iijima, M., Awala, S. K., Watanabe, Y., Kawato, Y., Fujioka, Y., Yamane, K., & Wada, K. C. (2016). Mixed cropping has the potential to enhance flood tolerance of drought-adapted grain crops. *Journal of Plant Physiology*, 192, 21–25. doi:10.1016/j.jplph.2016.01.004
Mashali, A. M. (1999). Land degradation with focus on salinization and its management in Africa. In H. Nabhan, A. M. Mashali, & A. R. Mermut (Eds.), *Integrated soil management for sustainable agriculture and food security in Southern and East Africa* (pp. 17–47). Rome: FAO.

Matusso, J. M. M., Mungwe, J. N., & Mucheru-Muna, M. (2014). Potential role of cereal-legume intercropping systems in integrated soil fertility management in smallholder farming systems of Sub-Saharan Africa. *Research Journal of Agriculture and Environmental Management, 3*, 162–174.

Mendelsohn, J., Obeid, S., & Roberts, C. (2000). *A profile of north-central Namibia* (pp. 18–19). Windhoek: Gamsberg Macmillan.

Mitsuya, S., Yano, K., Kawasaki, M., Taniguchi, M., & Miyake, H. (2002). Relationship between the distribution of Na and the damages caused by salinity in the leaves of rice seedlings grown under a saline condition. *Plant Production Science, 5*, 269–274. doi:10.1626/pps.5.269

Munns, R., James, R. A., & Läuchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany, 57*, 1025–1043. doi:10.1093/jxb/erj100

Munns, R., James, R. A., Xu, B., Athman, A., Conn, S. J., Jordans, C., ... Gilliham, M. (2012). Wheat grain yield on saline soils is improved by an ancestral Na+ transporter gene. *Nature Biotechnology, 30*, 360–366. doi:10.1038/nbt.2120

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology, 59*, 651–681. doi:10.1146/annurev.arplant.59.032607.092911

Ravindran, K. C., Venkatesan, K., Balakrishnan, V., Chellappan, K. P., & Balasubramanian, T. (2007). Restoration of saline land by halophytes for Indian soils. *Soil Biology and Biochemistry, 39*, 2661–2664. doi:10.1016/j.soilbio.2007.02.005

Silveira, J. A. G., Melo, A. R. B., Viégas, R. A., & Oliveira, J. T. A. (2001). Salinity-induced effects on nitrogen assimilation related to growth in cowpea plants. *Environmental and Experimental Botany, 46*, 171–179. doi:10.1016/S0098-8472(01)00095-8

Tavakkoli, E., Rengasamy, P., & McDonald, G. K. (2010). High concentrations of Na+ and Cl– ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. *Journal of Experimental Botany, 61*, 4449–4459. doi:10.1093/jxb/erq251

Willey, R. W., & Rao, M.R. (1980). A competitive ratio for quantifying competition between intercrops. *Experimental Agriculture, 16*, 117–125. doi:10.1017/S0014479700010802

Yamane, K., Rahman, M. S., Kawasaki, M., Taniguchi, M., & Miyake, H. (2004). Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.). *Plant Production Science, 7*, 292–300. doi:10.1626/pps.7.292

Yang, X., Liang, Z., Wen, W., & Lu, C. (2008). Genetic engineering of the biosynthesis of glycinebetaine leads to increased tolerance of photosynthesis to salt stress in transgenic tobacco plants. *Plant Molecular Biology, 66*, 73–86. doi:10.1007/s11103-007-9253-9