ENHANCEMENT OF SALINITY TOLERANCE OF COWPEA (*Vigna unguiculata*) BY MIXED CROPPING WITH ICE PLANT (*Mesembryanthemum crystallinum*)

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NANHAPO, PAMWENAFYE INATUTILA

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CERTIFICATION PAGE

I certify that this research work, **ENHANCEMENT OF SALINITY TOLERANCE OF COWPEA (*Vigna unguiculata*) BY MIXED CROPPING WITH ICE PLANT (*Mesembryanthemum crystallinum*)**, was carried out by **NANHAPO, Pamwenafye Inatutila**.

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DEDICATION

This study is dedicated to my loving wife,

Penehafo and our sons, ***Tukoleka*** & ***Moudiinini***

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ABSTRACT

The alleviative effect of mixed cropping using a salt accumulating halophytic ice plant (*Mesembryanthemum crystallinum*), to mitigate salinity damage and growth inhibition of cowpea (*Vigna unguiculata*), which is not tolerant to high salinity, was investigated in the greenhouse experiment. Cowpea is one of the most important legume intercrop in most cropping arrangements in the subsistence farming systems in arid and semi-arid regions of Sub-Saharan Africa. It is primarily cultivated for its pulses and used as a vegetable, livestock fodder, and is a source of cash for resource poor-farmers. More importantly, cowpea plays a major role in enriching the soil with nitrogen fixed in its root nodules in a symbiotic relationship with *Rhizobium* bacteria. In dry countries, for example Namibia, cowpea is mainly grown in the mixed cropping system with drought tolerant cereals such as pearl millet and sorghum, and other dryland crops to increase their productivity. Cowpea is considerably adapted to drought and high temperature; but it is not tolerant to high salinity. Ice plant is a salt-accumulating halophyte originated in the Namibian desert. It is primarily used for medicinal purposes and also consumed as a raw or cooked vegetable. Ice plant is very tolerant to salinity and drought, and has been used for desalination of salt affected soils. In this study, the mixed-seedling system, which is the planting of seedlings of

two crops in close proximity to enhance the intertwining of their roots, was used to investigate the potential of ice plant to mitigate salinity stress of cowpea.

Three cropping patterns (mono cropping of cowpea and ice plant and their combination) were tested under consecutive NaCl treatment, short-term recovery and long - term recovery experiments. In both experiments, ice plant was grown for 4 weeks prior to the sowing of cowpea. Pre-germinated cowpea seedlings were relay-planted in the cell where the seedlings of 28-day-old ice plant were grown (mixed cropping). All plants (three cropping patterns) were then grown in hydroponic boxes containing the full-strength Hoagland's solution for 14 days. In the consecutive NaCl experiment, plants were treated with 0, 100, 200 and 300 mM NaCl for 14 days (consecutive NaCl). In the recovery experiments, plants were treated with NaCl for 3 days, followed by 2 weeks recovery (short-term recovery) and 1-month recovery (long-term recovery). Salinity levels for short-term recovery were similar to those of the consecutive experiment, while those of long-term recovery were 0 and 250 mM.

The alleviative effects of mixed cropping in the consecutive NaCl experiment were observed at 200 and 300 mM NaCl. The decreases in the SPAD value, the photosynthetic rate, survival rate and the growth inhibition of cowpea mix-cropped with ice plant at 200 and 300 mM NaCl were suppressed compared with mono

cropping. 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. However, SPAD value, the photosynthetic rate and survival rate did not decrease under 100 mM compared with control (0 mM NaCl), while the content of Na^+ increased. These results suggested that 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. Mixed cropping was effective to recover from high concentration of NaCl in the experiments of short- and long-term recovery. In the long-term recovery experiment, the SPAD value and the photosynthetic rate of cowpea mix-cropped with ice plant recovered at similar level to that of the control after 1 month of recovery, while the physiological traits of mono-cropped cowpea did not recover fully. The regrowth was restored only in the mix-cropped cowpea. In addition, the shoot dry weight and relative growth rate values of mixed cropping after recovery from 250 mM NaCl were statistically higher than those of mono cropping.

These results indicated 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.

1. INTRODUCTION

1.1. Background

Soil salinity is one of the major abiotic stresses leading to low agricultural productivity in the arid and semiarid regions of the world. The Food and Agriculture Organization (FAO) estimated that more than 800 million hectares of the world's land is salt affected. In the arid and semiarid regions of Africa, more than 80 million ha of land area is affected by salinity (FAO and ITPS, 2015). With the combined effect of high temperatures and high rates of evapotranspiration, the annual rainfall in these regions is insufficient to meet plant evaporative demands, and to leach soluble salts and excess sodium ions from the rhizosphere (FAO and ITPS, 2015). In general, salinity reduces crop yields per unit area and hence the reduction of income to farmers (Munns and Gilliham, 2015).

The presence of salinity in agriculturally productive lands remains a substantial concern in the driest countries of Sub-Saharan Africa, for example Namibia, a dry country situated in south west Africa. In this country, where arable land area is limited (only about 1% of total land; Jones et al., 2013), salt-affected soils cover about 5.13 million ha of land (Mashali, 1999), which accounts for about 6 % of the total land area. Salt affected soils in Namibia are predominantly found in the arid and the most densely populated north central regions (Coetzee, 2003), where inhabitants' livelihood relies primarily on subsistence agro-pastoralism (Mendelsohn et al., 2002; 2013; Mendelsohn, 2006). These regions are the most populous due to the presence of seasonal wetlands that form part of the Cuvelai drainage basin (a shallow river system originating from the high rainfall area in the southern region of Namibia's northern neighbour, Angola).

These seasonal wetlands are prone to frequent occurring floods and droughts, causing a significant loss of local crop production. Flooding often occurs due to high rainfall in the upper catchment region of the Cuvelai basin (southern Angola). Moreover, the flood waters wash soluble salts from the upper region of the basin to north central Namibia. The recuring cycles of flooding and drought seasons coupled with high evaporation rates and capillary rise of salt containing ground water in these regions has led to the accumulation of soluble salts including sodium chloride (NaCl) (Zandler, 2011), which is the dominat salt in soils of these seasonal wetlands.

Mendelsohn et al. (2000) indicated that the highest salt content is found mainly in soils of the low laying areas and also in some soils of the higher grounds (upland); and only few salt insensitive plant species grow in these soils.

The rapid expansion of salt affected areas in productive agricultural lands needs an urgent advancement of the agricultural system to sustainably mitigate the adverse effects of salt stress on plant growth. Namibia, for example, with limited suitable land for crop production, any loss of the productive land to salinity (especially in the north central regions) will be practically detrimental and salinity will hinder any effort to increase crop productivity in these regions.

1.2. Effect of salinity stress on glycophytic plants

Salinity in the soil or in irrigation water is mainly elucidated when the quantity of soluble ions such as sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), magnesium (Mg^{2+}), chloride (Cl^-) or sulphate (SO_4^{2-}) ions exceeds a particular level. Of all soluble salts, NaCl is the most prevalent soluble salt in many saline soils and water.

According to Richards (1954), soils for which the electrical conductivity (ECe) of the saturation extract is more than 4 dSm^{-1} at 25 ºC are considered to be saline affected, because this salinity level causes 50% reduction in yields of most crops. In general, salt ions interfere with the normal physiological functions of plants. Salinity stress affects essential growth and developmental processes such as photosynthesis, protein synthesis, and energy and lipid metabolism (Parida and Das, 2005) hence reducing plant growth, development and survival (Carillo et al., 2011).

Previous studies indicated that plants are most sensitive to salinity stress during seedling emergence and early juvenile stages. For example, Mass (1993) indicated that cowpea *(Vigna unguiculata*), sorghum *(Sorghum bicolor* (L.) Moench) and wheat *(Triticum aestivum* L.) are most sensitive during their vegetative and early reproductive stages. Futhermore, Mass and Poss (1989) concluded that cowpeas were most sensitive to 20 days of salinity stress during their vegetative growth stage (7 to 27 days after planting, DAP) compared with flowering (27-47 DAP) and grain filling (52-72 DAP) stages.

The primary effects of salinity on glycophytic plants are osmotic stress and ion toxicity, resulting in oxidative stress.

Osmotic stress is caused by the presence of salt ions in a saline affected soil or growth solution that are exerting a very strong negative osmotic pressure, thus reducing the capacity of plant roots to absorb water (Morales et al., 2012). Osmotic stress causes physiological drought in glycophytes, preventing water uptake and interfere with nutrient transport in plants and this could be the main reason for growth inhibition (Türkan and Demiral, 2009; Munns, 2002). Physiological drought is observed immediately after salinity exposal (Munns, 2002; Tester and Davenport, 2003). Osmotic effect disturbs water balance in plants and lead to stomatal closure and reduced photosynthesis (Türkan and Demiral, 2009). Stomatal closure activates the production abscisic acid (ABA) in plant roots, which increases the production of reactive oxygen species (ROS) (Upadhyaya et al., 2013).

Ionic toxicity occurs when injurious salt ions i.e. Na^+, Cl^- or SO_4^{2-} accumulate in plant cells (Taiz and Zeiger, 2006; Morales et al., 2012), thus inhibiting many physiological and biochemical processes needed for growth and development in plants.

To date much work on salinity has focused mainly on the toxic effects of Na^+ and Cl^- ions. Of the two ions, Na^+ represents the major ion causing toxicity related with high salinity (Türkan and Demiral, 2009) because it appears to reach toxic concentration before Cl^- does (Ondrasek et al., 2011) and its extrusion mechanisms, which aid salt tolerance in plants have been extensively studied (Tester and Davenport, 2003).

Plants experience ionic stress during long-term exposal to salinity stress. Salt ions accumulate in plants and reach toxic levels, and eventually injure the transpiring leaves (Munns, 2005). Specific damage of plant cells and tissues depends on the rate of ion (Na^+) accumulation and the effectiveness of compartmentation within leaf tissues and cells (Tester and Davenport, 2003). Na^+ ionic stress affect plant metabolic activities by disrupting protein synthesis and interfere with enzyme activity, which leads to premature senescence, chlorosis, necrosis and even death of older leaves. As a result, the photosynthetic capacity of older leaves may no longer be able to support carbohydrate requirements for the young leaves (Munns and Tester, 2008).

The metabolic toxicity of Na^+ is mainly due to its ability to compete with K^+ for binding sites essential for cellular function (Tester and Denver, 2003). K^+ regulate osmotic potential and play a major role in the activation of many crucial enzymatic reactions needed for respiration and photosynthesis in plants (Taiz and Zeiger, 2006).

Na^+ could easily replace K^+ in plant cells due to the physicochemical similarities of their structures (Maathuis and Amtmann, 1999). Therefore, excess Na^+ ions inhibit metabolic processes by competing and substituting K^+ at the binding sites of many essential enzymes that are activated by K^+. However, the functions of K^+ cannot be substituted by Na^+, thus higher level of Na^+ or high Na^+/K^+ ratios can disrupt various enzymatic processes in the cytoplasm and inhibit protein synthesis (Tester and Davenport, 2003).

1.3. Strategies to overcome salinity stress in plants

A wide range of adaptations and mitigation strategies are required to cope with salinity stress in plants (Shrivastava and Kumar, 2015). Plant adaptive mechanisms include 1) exclusion of salt ions, the reduction of Na^+ accumulation in cytosol cells, Na^+ compatmentation in cell vacuoles; 2) changes in membrane structure to control ion uptake by roots and transport into leaves; 3) induction of antioxidative enzymes and the synthesis of osmolytes for osmotic adjustment; and 4) genetic engineering of salt tolerance in plants (Rajendran et al., 2009; Carillo et al., 2011; Parida and Das, 2005). In addition, inoculation with microbes (Rabie et al., 2005; Carmen and Roberto, 2011) was also reported to increase salinity tolerance. Another technique that contributes to reduced Na^+ in leaves is grafting (i.e. Albacete et al., 2009). The authors reported using tomatoes that the concentration of Na^+ 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.

Although there are many strategies to increase salt tolerance, the ability to minimize the amount of Na^+ accumulation in photosynthetic leaves is one of the key factors to improve salt tolerance (Carillo et al., 2011). One of the key factors to improve salt tolerance is reducing the amount of Na^+ transported from roots to shoots

(Munns & Tester, 2008), for which a few techniques have been proposed. The major technique would be identifying the genes related to Na^+ transporters and introducing them into salt-sensitive plants for improving salt tolerance.

Chinnusamy et al. (2005) have indicated the usefulness of the plasma membrane embedded Na^+/H^+ antiporter, SOS1, in transgenic *Arabidopsis sos* mutants to extrude Na^+ from root epidermal cells under salinity when compared with wild types. Moreover, Munns et al. (2012) reported that the gene *TmHKT1;5-A* in the *Nax2* locus encodes the Na^+ - selective transporter located on the plasma membrane of root cells surrounding xylem vessels, and this transporter contributes to withdrawal of Na^+ from xylem cells, thus reducing the amount of Na^+ in the leaves of durum wheat. The durum wheat exhibited 25% higher yield than the near-isogenic line without the *Nax2* locus in a field condition.

Phyto-amelioration, which is the method for the removal of excess salt from soils using salt-adapted plants, was also proposed (Ammari et al., 2008; Ravindran et al., 2007).

1.4. Mixed cropping alleviates stress of stress intolerant crops

Several low-technology agronomic approaches including mixed cropping could be used to improve crop productivity under stress conditions (Carmen and Roberto, 2011) including salinity stress (Ondrasek et al., 2011), especially in resource poor farmers' fields.

Mixed cropping is one of the cheapest (low-tech) and the oldest method of crop farming practiced by many farmers in the Sub-Saharan Africa region, because it is sustainable and has high returns with minimal input. Mixed cropping involves the planting of two or more crops that will grow in a complementary association for the efficient use of natural resources. Despite the effect of competition for the resources such as solar radiation, mineral nutrients, water, etc. among the component crops in the mixed cropping system, they often benefit each other in various aspects including stress alleviation.

Legumes are one of the common component crops in mixed cropping system. They are often grown in combination with cereals and other crops in order to augment the nitrogen availability for the non – leguminous crops. For instance, growing chickpea with wheat, or sorghum + pigeon pea + cowpea combination (Chandrasekaran et al., 2010); cowpea + upland rice (Okereke and Ayama, 1992); etc.

Therefore, a legume + cereal mixed cropping alleviates N starvation of cereals.

Recent studies have reported on the benefits of mixed cropping to alleviate stress of stress sensitive plants by planting in close proximity with stress tolerant plants under stress conditions. For example, Iijima et al. (2016) reported that planting rice (flood tolerant) in a mixed cropping system with flood intolerant millets (pearl millet or sorghum) has alleviated flooding stress of the millets under flooding conditions in the glasshouse. The authors indicated that rice might have enriched the rhizosphere with oxygen released from its oxygen storage chambers in its roots (aerenchyma) through the process commonly known as radial oxygen loss (ROL). The millets may have utilized the oxygen released from rice in mixed cropping. The growth of flood intolerant millets in mixed cropping was facilitated in comparison with mono cropped millets under flooding stress. Moreover, rice alleviated flooding stress of millets in mixed cropping system under flooding conditions in the field (Awala et al., 2016). This study was performed under controlled flooding condition in the field in the semi-arid north central Namibia. The authors reported that under flooding conditions, mixed cropping increased plant survival rates and land equivalent ratios of both pearl millet and sorghum, and minimized grain yield loss of millets in mixed cropping compared with mono cropped millets.

1.5. Salinity mitigation for cowpea by mixed cropping with ice plant

Cowpea (*Vigna unguiculata*), a moderate salt tolerant legume (Murillo-Amador et al., 2006), is one of the most important legume intercrop in most cropping arrangements in the subsistence farming system in arid and semi arid regions of the SSA region and more than three-fourth of the production area is spread in the region. In these regions, it is primarily cultivated for its edible pulses and used as a vegetable, livestock fodder (Dumet et al., 2008) and is a source of cash for resource poor-farmers (Olufajo, 2012). In addition, cowpea plays a major role in enriching the soil with nitrogen fixed in its root nodules in a symbiotic association with *Rhizobium* bacteria and through green manure. In Namibia for example, cowpea is mainly grown in the mixed cropping system with drought tolerant cereals such as pearl millet and sorghum, and other dryland crops to increase productivity. Cowpea is considerably adapted 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, salinity stress strongly inhibits its growth and yield primarily due to the accumulation of Na^+ and Cl^- in the leaves and a decrease K^+ and K^+/Na^+ ratio (Cavalcanti et al., 2004; Taffouo et al., 2009; Gogile et al., 2013). Ion accumulation in leaves significantly reduces chlorophyll content, photosynthetic rate, growth and the assimilation rate of cowpea. 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 cultivation of crops under saline conditions led to the rediscovery of halophytic species with greatest economic potential as vegetable crops (Ventura and Sagi, 2013; Herppich et al., 2008) and to provide fodder, fuel wood, industrial raw materials and income to farmers in salt affected lands (Hasanuzzaman et al., 2014).

The common ice plant (*Mesembryanthemum crystallinum*) is a salt-accumulating halophyte (Abd El-Gawad and Shehata, 2014) originated in the Namibian desert on the western coast of southern Africa (Bohnert and Cushman, 2000). It belongs to Aizoaceae family and is extremely stress tolerant (Abd El-Gawad and Shehata, 2014). Ice plant leaves and stems are utilised as a raw or cooked vegetable (Bohnert and Cushman, 2000; Herppich et al., 2008, Abd El-Gawad and Shehata, 2014).

Apart from its food uses, *M. crystallinum* plant extracts are rich in antioxidants and anti-microbial compounds which are principally used for medicinal purposes including the treatment of ascites, dysentery, liver and kidney diseases (Abd El-Gawad and Shehata, 2014, Deters et al., 2012; Ibtissem et al., 2012; Hanen et al., 2009). Agarie et al. (2009) indicated that salinity and drought stress induce the accumulation of polyols (compatible solutes) such myo-inositol, pinitol and anonitol for osmotic adjustment in ice plant. D-pinitol is used as a food supplement because of its insulin like function which is used for the treatment of diabetic neuropathy, depression, panic disoder and respiratory distress syndrome.

Ice plant has been used in desalination of salt affected soils (Hirokane et al., 2014). 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), which could be useful for bioremediation of saline affected soils (Bohnert and Cushman, 2000). Excess Na^+ is sequestrated in specialised epidermal bladder cells (EBC) (Adams et al., 1998; Agarie et al., 2007). EBCs are typically 500 μm in diameter and participate in the regulation of salt sequestration and water relations, and serve as a storage reservoir for water, organic and inorganic compounds including NaCl, thus play an important role in the salinity tolerance of ice plant (Agarie et al., 2007). Under salinity stress conditions, ice plant accumulates Na^+ in cell vacuoles for efficient osmoregulation of the cell sap and to maintain efficient water uptake and utilisation (Kholodova et al., 2002), thus it can thrive on soils containing NaCl concentration of 500 mM (Adams et al., 1998).

Ice plant is a facultative halophyte that shifts from C_3 photosynthetic pathway to Crassulacean Acid Metabolism (CAM) pathway once exposed to salinity stress (Bloom, 1979; Adams et al., 1998; Kholodova et al., 2002; Agarie et al., 2007). The salinity stress adaptive mechanisms of ice plant and its ability to shift to CAM photosynthetic pathway under stress makes it to be one of the candidate halophytes for cultivation in salt affected soils in the arid and semi arid regions.

Both ice plant and cowpea are adapted to high temperature, low rainfall and low nutrition that are prevalent in the arid and semi arid regions. The mixed cropping system using ice plant might reduce the ion content in the leaves of salt-sensitive crops of drier tropical regions such as cowpea and mitigate the damage and growth inhibition. Moreover, crop farmers will harvest the produce from both crops of the mix cropping system for consumption and/or to generate income, which will improve their livelihoods at low production input.

1.6. Scope and objectives of the study

This study focuses on the potential of the edible halophytic ice plant (*M. crystallinum*) to mitigate salinity stress in cowpea when the two crops are planted together in the mixed cropping system. Recent studies indicated that mixed cropping with the flood tolerant plant of rice under flooding conditions enhanced the growth, physiological traits and the survival rate of flood sensitive plants such as pearl millet and sorghum in glasshouse and in field, probably because of transferring oxygen from rice to pearl millet or sorghum (Iijima et al., 2016; Awala et al., 2016).

These abovementioned studies lead us to the hypothesis that the salt tolerance of salt sensitive plants could be improved by mixed-cropping with a salt accumulating halophyte because it would preferentially absorb and accumulate salt in the body, leading to 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. However, literatures on the utilization of salt accumulating halophytes to mitigate salinity stress of cowpea, when the two crops are grown together in one hill are limited.

The aim of the present study was to evaluate the physiological and growth responses of cowpea when mix planted with a salt accumulating ice plant compared with cowpea single crop under salinity. Therefore, 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 in cowpea leaves. We further discuss the usefulness of the mixed cropping system to alleviate salinity stress.

2. MATERIALS AND METHODS

2.1. Plant materials

A series of experiments were conducted in the greenhouse at the experimental filed of Faculty of Agriculture, Kindai University, Nara (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, Omahenene, 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. Cowpea and ice plant were grown 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 (H_2O) 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 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 to allow the intertwining of their roots. 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. The part of cell trays below the soil surface was covered with polyvinyl sheets to prevent from light penetrating.

NaCl salt stress was imposed to the mono- and mix-cropped cowpea plants after 14 days of growing in hydroponic boxes. According to Mass and Poss (1989), cowpea is most sensitive to salinity stress at this growing stage. At the imposition of salinity stress, cowpea plants were 14-day-old, and the second trifoliate leaves were fully expanded, while ice plants were 42-day-old, and the growth stage was late juvenile (4 leaf stage), which was judged according to Adams et al. (1998). The epidermal bladder cells (EBC) on the stems and leaves of ice plant at this growth stage become more turgid and protruding for salt storage, which may play a crucial role in salinity mitigation for the companion crop. 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 solutions were 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 also 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 3 days of salinity treatment, the plants were transferred to 1/5000 a Wagner pot for 1 month recovery. In this study, sandy loam soil with a pH (H_2O) of 5.65, EC of 0.80 ms m^{-1}, total N of 0.03 g kg^{-1} and total C of 0.46 gkg^{-1} was used. During the recovery period, the plants were watered every third day with full-strength Hoagland's solution without NaCl. The treatments were arranged in a randomised complete block design with three replicates, and six plants were included in each replication.

2.2. Measurements of physiological traits and growth parameters

The leaf greenness and the photosynthetic rate (Pr) of the uppermost fully expanded trifoliate leaves of mono- and mix-cropped cowpea were measured using the SPAD (soil plant analysis development) chlorophyll metre (SPAD-502; Minolta 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 CO_2 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. SPAD values and photosynthetic rates were monitored on day 3, 6, 9 and 12 of salt treatment in the consecutive NaCl and on similar day-intervals of salinity recovery in the short-term recovery experiments. In the long-term recovery experiment, the physiological traits 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 (Pr). 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 shoot dry weight measurement. Shoot relative growth rate (RGR) was estimated as the difference in dry weight over time interval (g g^{-1} d^{-1}) and was calculated using the following equation according to Hoffman and Poorter (2002):

$$RGR = (\overline{\ln W_2} - \overline{\ln W_1}) / (t_2 - t_1)$$

where $\overline{\ln W_2}$ and $\overline{\ln W_1}$ 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.

The relative yield (RY) and relative yield total RYT at each salinity level (0, 100, 200 and 300 mM NaCl) were computed to evaluate the advantage of the mixed cropping system compared with mono cropping. The RY of a crop component in the mixed cropping system was determined as a yield of that crop in the mixed cropping expressed as a proportion of its yield in mono-cropping. The RYT was calculated as the sum of RY of component crops in mixed cropping. RY and RYT were calculated using the following method by Caballero et al. (1995) and Keddy et al. (1994):

RY (cowpea) = Yield of cowpea in the mixed cropping/ Yield of cowpea in mono cropping

RY (ice plant) = Yield of ice plant in the mixed cropping/ Yield of ice plant in mono cropping

Where "Yield" is shoot dry weight at the end of the experiment.

RYT = RY (cowpea) + RY (ice plant)

RY > 0.5 indicates a positive effect of the mixed cropping system on yields, and RY < 0.5 indicates the disadvantages by the mixed cropping; while RYT > 1 indicates positive effect of the mixed cropping , and RYT < 1 indicates a disadvantage of the mixed cropping (Tarui et al., 2013).

A competitive ratio (CR) measures the competitive ability between different crop components in the mixed cropping system. CR was calculated using the shoot biomass production of the two component crops following the method of Willey and Rao (1980):

$$CR\ (Cp) = (Y_{Cp\text{-}mix}/Y_{Cp\text{-}mono}) / (Y_{Ip\text{-}mix}/Y_{Ip\text{-}mono})$$

$$CR\ (Ip) = (Y_{Ip\text{-}mix}/ Y_{Ip\text{-}mono})/(Y_{Cp\text{-}mix}/Y_{Cp\text{-}mono})$$

where Y is the shoot dry weight of the plant. When CR > 1, the mixed cropping favors the growth and yield of crops. When CR < 1, the mixed cropping negatively affects the growth and yields in mixture.

2.3. Survival and recovery rate

The effect of NaCl treatment on survival rate was calculated using the following equation: survival rate (%) = (n / 6) x 100, where n is the number of surviving plants of total 6 plants in each replication. Survival rate was monitored at 2 day interval during the 14 days of salinity treatment in the consecutive NaCl and on similar day-intervals of salinity recovery in the short-term recovery experiments. Plant survival was based on the visual observations.

2.4. Na^+ and K^+ content determination

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 homogenised 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) 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.

2.5. Statistical analysis

The statistical significance between cowpea mono and mixed cropping in Fig. 1, 2, 3, 5, 6, 7 and 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, Fig. 4 and Fig. 11, 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. Survival rate percentage data of cowpea mono and mixed cropping in Fig. 6 and 10 were compared by independent samples *t*-test.

3. RESULTS

3.1. 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 (Fig. 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 (Fig. 1, left upper). The SPAD values of mono and mixed cropping under 100 mM NaCl were maintained around 50 (Fig. 1, right upper), 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 (Fig. 1, lower). The values after the treatment with 200 and 300 mM NaCl for 12 days were 17.2 (Fig. 1, left lower) and 0 (Fig. 1, right lower), respectively. Mixed cropping using ice plant was effective in mitigating the decrease in SPAD values caused by the high concentration of NaCl (Fig. 1, lower). The SPAD value of mix-cropped 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 $m^{-1}s^{-1}$, and the cropping pattern did not influence the photosynthetic rates (Fig. 2, left upper). 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 (Fig. 2, right upper). Fig. 2 lower 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.

Fig. 3 shows Na^+ and K^+ content in the leaves of cowpea and ice plant of control and after treatments with 100, 200 and 300 mM NaCl after 14 days of salinity treatment. The treatments with 100, 200 and 300 mM NaCl reduced the K content in the leaves of cowpea (Fig. 3, left upper) and ice plant (Fig. 3, right upper) 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 (Fig. 3, left upper). 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 (Fig. 3, right upper). The Na^+ content in the leaves of cowpea and ice plant treated with salt stresses increased with increasing NaCl concentration supplied to the plants (Fig. 3, middle). 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 were lower than that of mono cropping, and a statistical difference was evident for cowpea (Fig. 3, left middle). The lower Na^+ content of cowpea mixed cropping exhibited a statistical decrease in Na/K ratio at 200 and 300 mM NaCl (Fig. 3, left lower). 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 (Fig. 3, right lower), which is induced by the higher K^+ content in each treatment.

Fig. 4 shows the soil Na^+ content of mono and mix-cropped cowpea and mono-cropped ice plant 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 treated with 200 and 300 mM NaCl was statistically lower than that of mono-cropped cowpea.

Fig. 5 upper 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. Fig. 5 lower 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 mix-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 2.00, indicating the successful combination in terms of mixed cropping.

Fig. 6 shows the survival rate of cowpea mono cropping and cowpea mixed cropping during the 14 days under continuous 100, 200 and 300 mM NaCl salinity treatment. The average survival rate was 100% in the control and in 100 mM NaCl treatment (Fig. 6. upper right and left). The survival rate under 200 and 300 mM treatment decreased after day 6 of salinity treatment in both cowpea mono cropping and cowpea mixed cropping. Under 200 mM treatment, the survival rate of cowpea mono cropping was 53% at the end of the experiment (at 14 days of salinity treatment), which was significantly lower than cowpea mixed cropping which had 94% survival rate. However, under 300 mM, all cowpea mono cropping plants were completely killed by salinity (0% survival rate) by day 10 of salinity stress, while the average survival rate for cowpea mixed cropping was 44% at the end of the experiment.

The results indicated that mixed cropping was effective only at 200 and 300 mM, but not at 100 mM. Under 200 and 300 mM, cowpea mixed cropping showed a gradual decline in survival rate compared with cowpea mono cropping which indicated a very sharp death rate after day 6 of salinity stress.

3.2. Alleviative effects of mixed cropping after short-term recovery from different concentrations of salt stress

Fig. 7 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. In addition, the SPAD values of cowpea of mono and mixed cropping after the recovery from 100 (right upper) and 200 mM NaCl (left lower) were similar in both the cropping patterns. However, the SPAD values of mono-cropped cowpea after recovery from 300 mM NaCl rapidly decreased and the value reached almost 0 after 12 days of recovery (left lower). 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.

Fig. 8 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 (left upper). 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 (right upper) and 200 mM NaCl (left lower) 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 (right lower). 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 (right lower).

Fig. 9 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 mix-cropped 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 (left upper). The difference observed after the recovery from 100 mM NaCl was statistically significant (left upper). However, an opposite trend was observed in cowpea after recovery from 200 and 300 mM NaCl.

The shoot dry weights of mix-cropped cowpea after recovery from 200 and 300 mM NaCl were higher than those of mono cropping, and the difference observed with 300 mM NaCl was statistically significant (left upper). 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 (right upper). The

RGR values of cowpea and ice plant showed a similar trend as observed in the shoot dry weight (Fig. 9 lower). Statistically significant differences between mono and mixed cropping were not observed in all treatments.

Fig. 10 shows the survival rate of cowpea mono cropping and cowpea mixed cropping during 14 days of recovery from 3 days of salinity stress under 100, 200 and 300 mM NaCl treatment in short- term survival experiment. All cowpea plants under cowpea mono cropping and cowpea mixed cropping survived 100 mM NaCl stress. Infact, the survival rate was 100% at this level (100 mM) and mix cropping did not have any effect on the survival rate of cowpea during the 14 days of recovery from 3 days salinity stress. The survival rate under 200 mM (Fig. 10 bottom left) the survival rate was 100 % for both cowpea monocroping and cowpea mixed cropping until day 8. However, after day 8 of recovery until the end of the experiment the survival rate was 76% and 94% for cowpea mono cropping and cowpea mixed cropping, respectively.

Furthermore, the survival rate of cowpea monocropping under 300 mM NaCl (Fig. 10 bottom right) begin to decline steeply after day 3 of recovery and all crops succumbed to a 3 day salinity stress by day 10 of recovery and could not recover, while the average survival rate of cowpea mixed cropping was significantly higher than that of cowpea mono cropping from day 6 until the end of the experiment.

The 3-day exposal to 300 mM salinity stress caused excessive damage to cowpea mono cropping shoots, thus no plant survived and no re-growth by end of the experiment. However, more than 60% of cowpea mix cropping plants survived the 3 days 300 mM salinity exposal. Mixed cropping could have prevented salinity damage of cowpea mixed cropping.

3.3. 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). Fig. 11 left shows the time course of the SPAD values of mono- and mix-cropped cowpea of 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, 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.

Fig. 11 right shows the time course of the photosynthetic rates of cowpea in mono and mixed cropping of control and recovery from 250 mM NaCl. The photosynthetic rates of mono-cropped cowpea of control (white circle) ranged from 15 to 20 μmol m^{-2} s^{-1} during the experiment. The photosynthetic rates of mix-cropped cowpea of control (open triangle) were statistically lower than those of mono cropping, and the value was about half at day 0.

The low photosynthetic rates of mix-cropped cowpea should have been induced by competition with the ice plant. 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 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 (Fig. 12). Fig. 12, left, shows the shoot dry weight of mono- and mix-cropped cowpea. The shoot dry weight of mix-cropped cowpea of control was statistically lower than the mono-cropped plants. However, an opposite trend was observed in the cowpea recovered from 250 mM NaCl. The shoot dry weight of mix-cropped cowpea after the recovery was statistically higher than that of mono cropping with NaCl treatment.

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. On the other hand, the RGR value of mix-cropped cowpea after the recovery was statistically higher than that of mono cropping. Fig. 12, upper right, 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. Fig. 12 (lower right) 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.

The survival and recovery responses of cowpea mono cropping and cowpea mixed cropping under salinity treatment (250 mM) was evaluated to determine the effectiveness of mixed cropping under long-term recovery experiment. Branches and leaves of cowpea mono cropping became yellow and were shed after day 6 of the recovery period. However, 78% of plants recovered gradually and new trifoliates sprouted only from day 15 of the salinity recovery period onwards, while 22% of plants did not recover (completely killed by 3 days 250 mM NaCl treatment).

The 3-day salinity stress did not cause any serious damage to cowpea mixed cropping. Apart from the slow growth after salinity treatment, all cowpea mixed cropping plants retained their leaves and recovered very well from salinity stress. No yellowing of leaves, nor dead plants of cowpea mixed cropping were recorded (100% survival rate) until the end of the experiment. Furthermore, cowpea mixed cropping plants maintained their leaf greenness throughout the recovery period (Fig 11. left).

These results showed that mixed cropping could have reduced the absorption of Na^{+} by cowpea mixed cropping plants, hence reducing the salinity damage of plants, thus enhanced the survival of plants and promoted a significant recovery from salinity stress.

4. DISCUSSION

4.1. 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 of both cowpea mono cropping and mixed cropping 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 (Fig. 1, upper right). 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 (Fig. 3, left middle).

Also, the survival rate of cowpea mono and mixed cropping at 100 mM was 100% during the entire consecutive salinity treatment (Fig. 6 upper right). 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 (Fig. 1, lower) were lower than those of the control or at 100 mM NaCl (Fig. 1, upper), 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. 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.

The Na^+ contents in the leaves and soil of cowpea mix-cropped with ice plant at 200 and 300 mM NaCl significantly decreased compared with those in mono cropping (Fig. 3, left middle and Fig. 4), resulting in the lower Na^+/K^+ ratio (Fig. 3 left lower). 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 compartmentalization 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 is due to 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 (Fig. 4).

Moreover, cowpea mixed cropping showed a significantly higher survival rate under 200 and 300 mM NaCl stress compared with cowpea mono cropping. The survival rate could measure the severity of salinity damage to plant populations, in which cowpea mono cropping was severely killed by 200 and 300 mM NaCl treatment compared with cowpea mixed cropping. 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 alleviation of shoot biomass production of cowpea observed in the treatment with 200 and 300 mM NaCl could primarily be due to the decrease in the osmotic effects of salinity, judging from the data of the SPAD values, photosynthetic rates and Na^+ contents in soil and leaves, 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 (Fig. 5) at both 200 and 300 mM NaCl, probably because of the higher photosynthetic rates (Fig. 2). The decreased rate of shoot growth under salinity in cowpea mono cropping 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). The leaf (Fig. 3 middle left) and soil Na^+ (Fig. 4) contents in mix-cropped cowpea at 200 and 300 mM were statistically lower than those of mono-cropped cowpea (Fig. 4).

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^+ content.

4.2. 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 under salt stress 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 photosynthetic rate was lower than that of other treatments (Fig. 11).

On the other hand, the physiological activity of the new leaves of mix-cropped cowpea was similar to that of the control (Fig. 11). In the mono-cropped cowpea, the uppermost fully expanded leaves treated with 250 mM NaCl wilted during the recovery. However, the uppermost fully expanded leaves in mix-cropped cowpea did not wilt and the photosynthetic rate rapidly recovered after the recovery (Fig. 11, right).

The shoot dry weight and RGR values of mixed cropping after recovery from

250 mM NaCl were statistically higher than those of mono cropping (Fig. 12). These results suggest that maintenance of the photosynthetic rate in the uppermost fully expanded leaves during salt stress 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 leaf damage caused by salt stress.

4.3. 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 (Herppich et al., 2008, Abd El-Gawad and Shehata, 2014) 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 (Matusso et al., 2014; Dugje et al., 2009).

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 (Fig. 12, right upper). 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.

The growth of cowpea under control and moderate salinity (100 mM NaCl) was suppressed by the competition (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, 14 and 3 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.

5. FIGURES AND TABLE

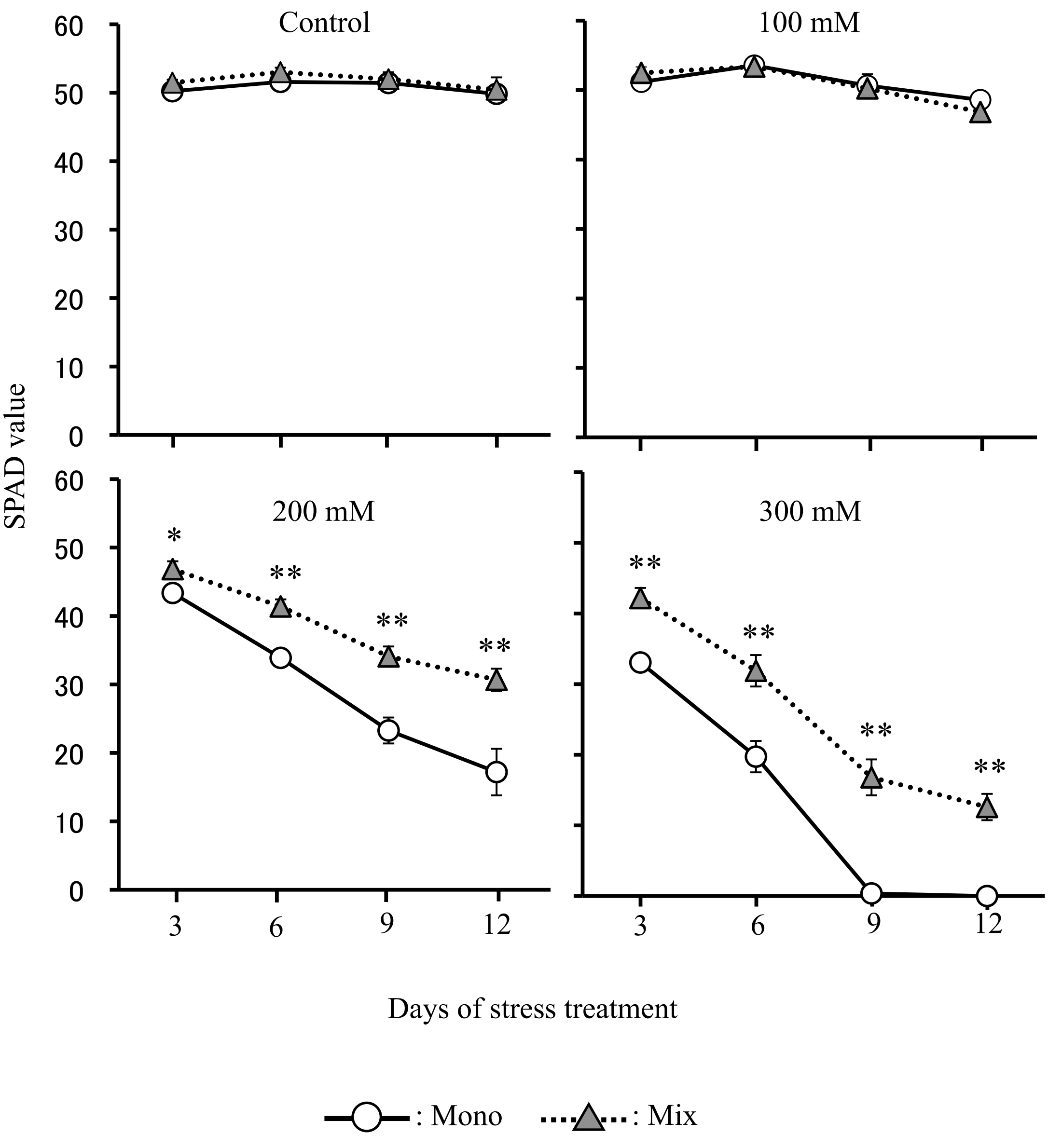

Fig. 1. Time course of the SPAD value of cowpea under 0, 100, 200 and 300 mM NaCl in the consecutive NaCl treatment experiment. The data are means ± SE (n = 6). The symbols of * and ** in each figure indicate the significant difference between mono and mixed cropping at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

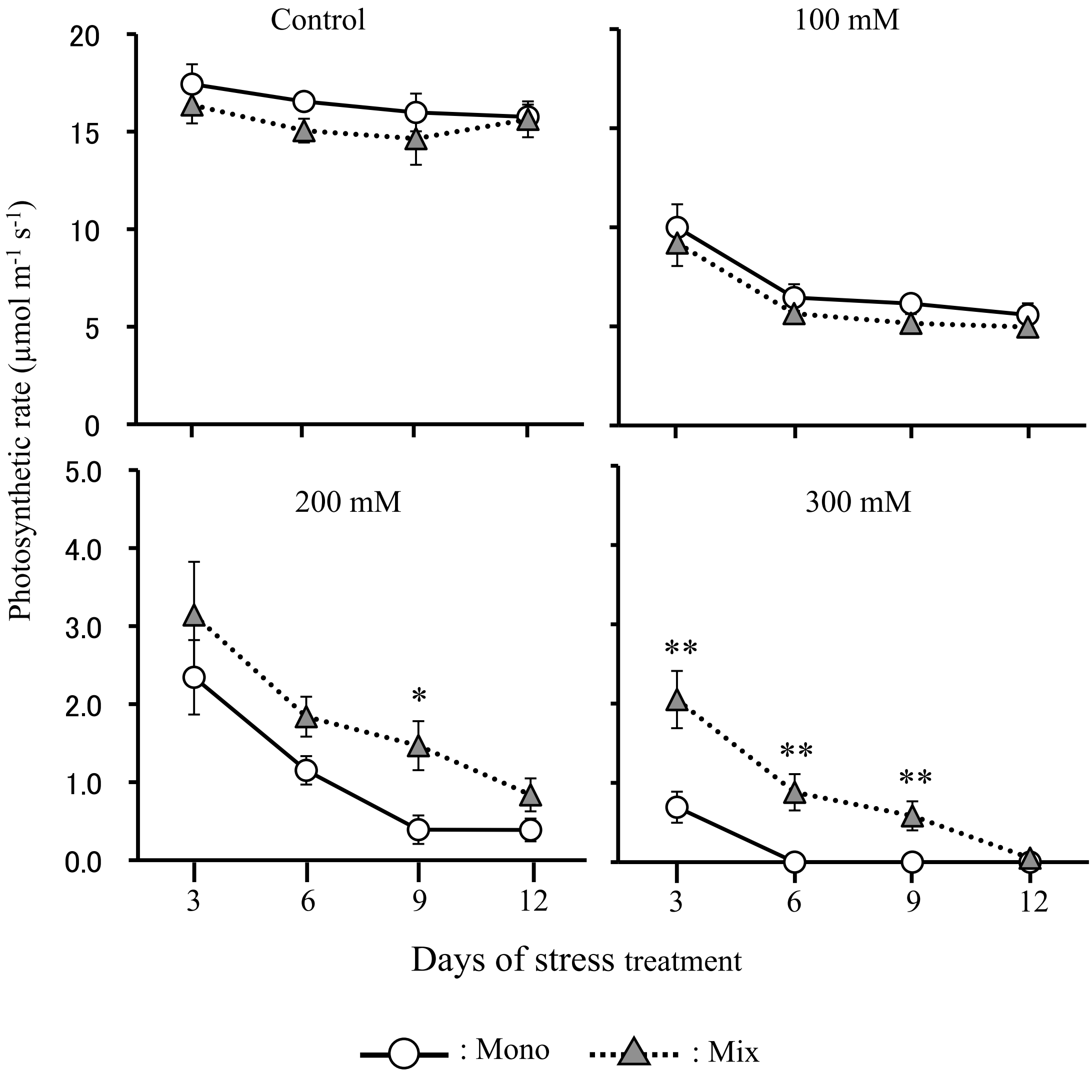

Fig. 2. Time course of the photosynthetic rate of cowpea mono and mixed cropping under 0, 100, 200 and 300 mM NaCl. The data are means ± SE (n = 6). The symbols of * and ** indicate the significant difference between mono and mix cropping in each day at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

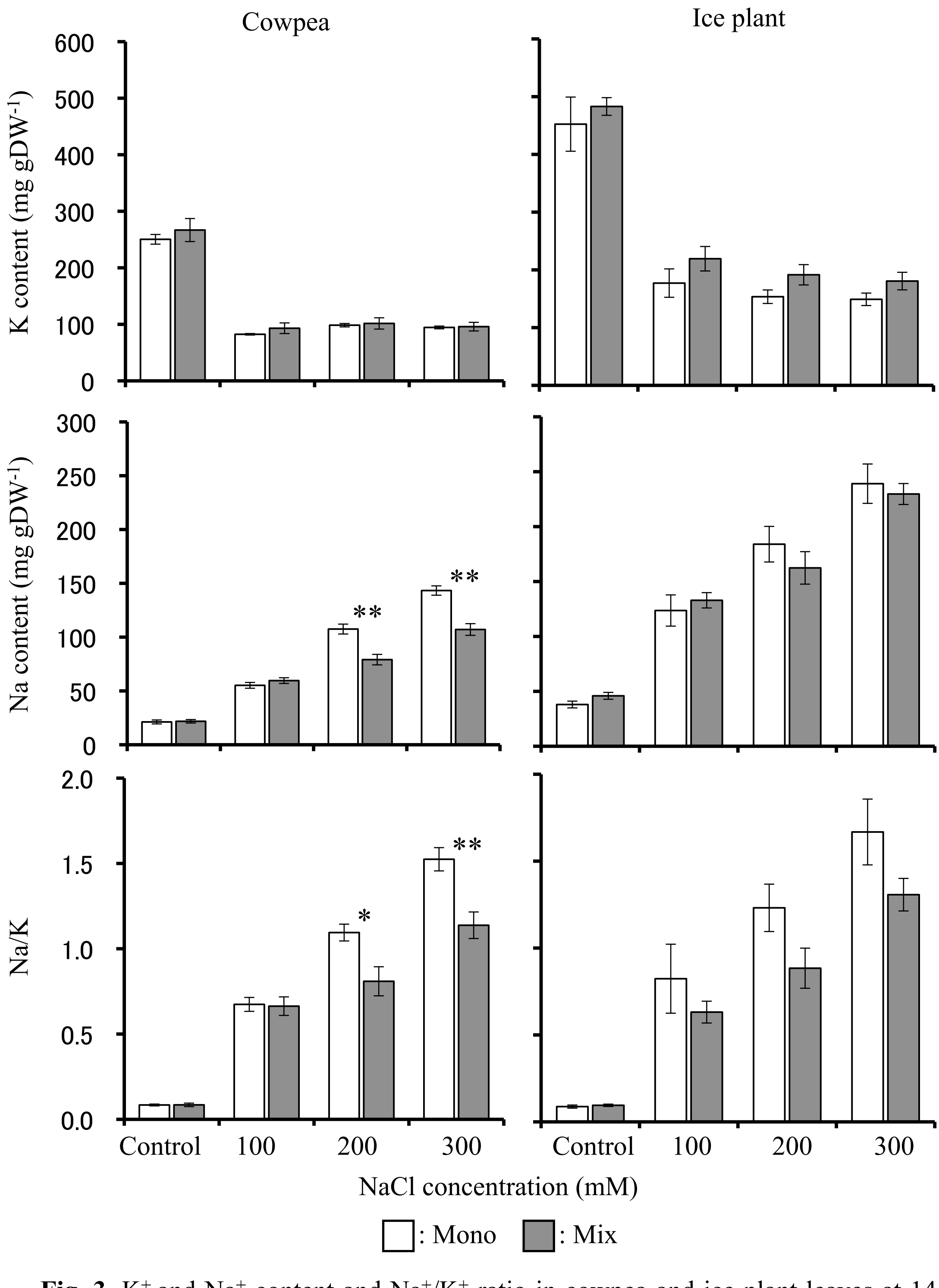

Fig. 3. K^+ and Na^+ content and Na^+/K^+ ratio in cowpea and ice plant leaves at 14 days after the treatment with 100, 200 and 300 mM NaCl. The data are means ± SE (n = 6). The symbol of * and ** indicate the significant difference between mono and mixed cropping in each NaCl concentration at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

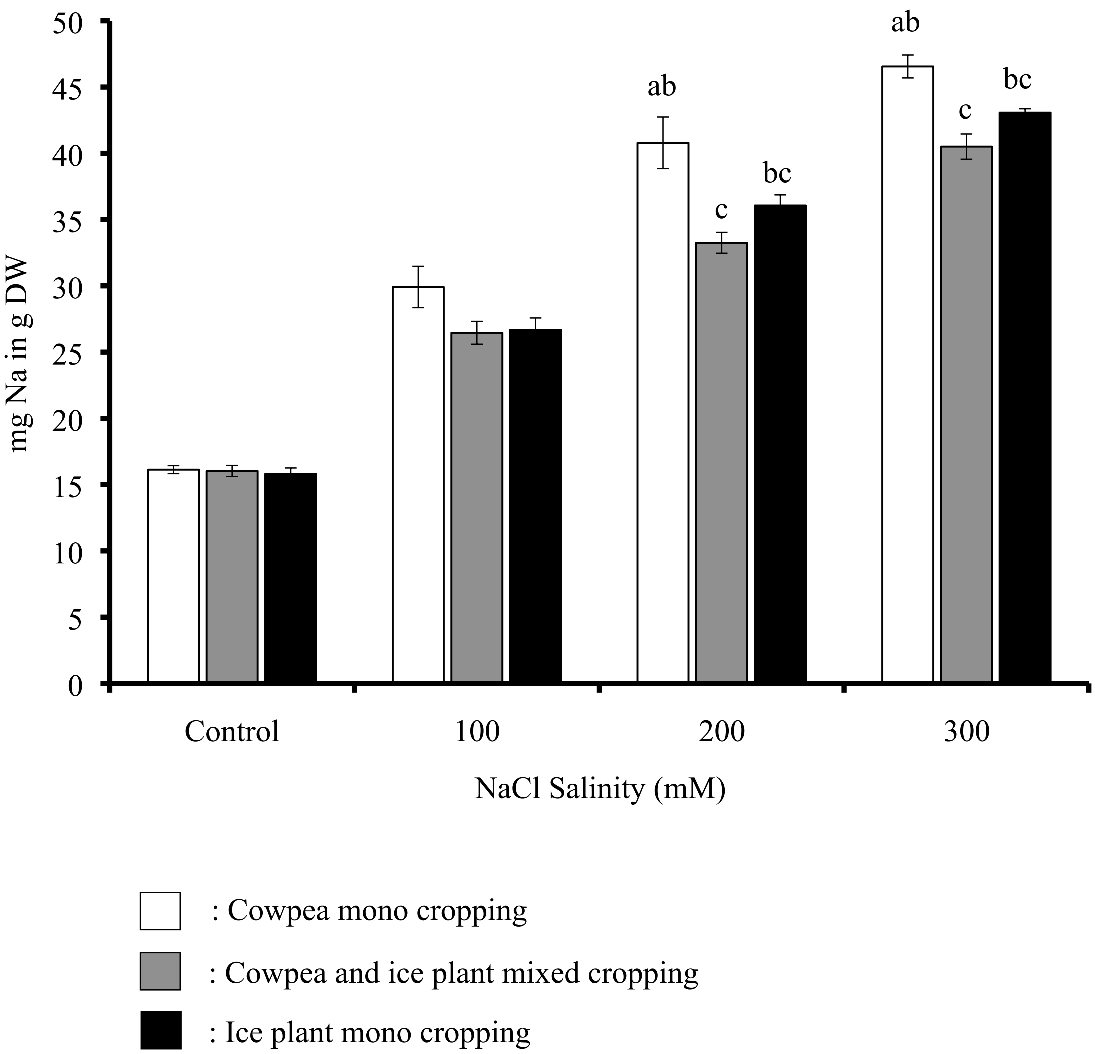

Fig. 4. Soil Na^+ content under mono and mixed cropping at 14 days after treatment with 100, 200 and 300 mM NaCl in the experiment of consecutive NaCl treatment. The data are means ± SE (n = 6). Different letters above the bars indicate significant differences at P<0.01 by Tukey-Kramer multiple comparison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

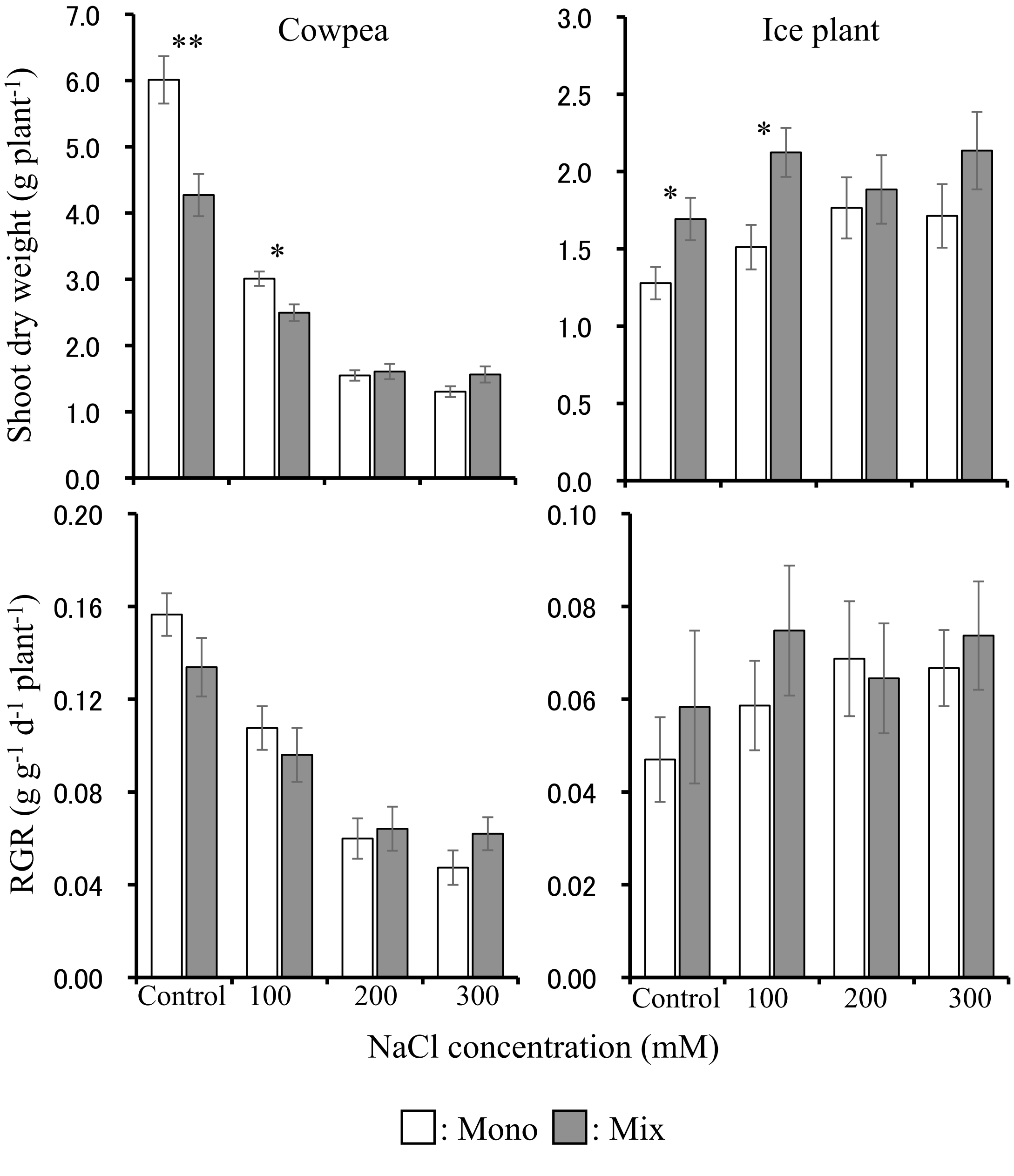

Fig. 5. Shoot dry weight and relative growth rate (RGR) of cowpea and ice plant of mono and mixed cropping at 14 days after the treatment with 100, 200 and 300 mM NaCl. The data are means ± SE (n = 6). The symbols of * and ** indicate the significant difference between mono and mixed cropping in each NaCl concentration at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

Table 1. Relative yield (RY), relative yield of total (RYT) and competitive ratio (CR) of cowpea and ice plant in the experiment of consecutive NaCl treatment.

NaCl concentration (mM)		RY (Cowpea)	RY (Ice plant)	RYT	CR (Cowpea)	CR (Ice plant)
0		0.72[a]	1.37	2.09	0.55[a]	1.94[a]
100		0.84[a]	1.46	2.30	0.60[a]	1.77[a]
200		1.04[b]	1.09	2.13	0.99[b]	1.05[b]
300		1.20[b]	1.25	2.44	0.96[b]	1.05[b]
One-way ANOVA	*F* value	15.31	1.490	1.106	10.79	8.158
	Probability	$2.08\times10^{-5++}$	0.248	0.370	$1.97\times10^{-4++}$	$9.62\times10^{-4++}$

The data are means of six replications. The symbol of ++ indicate significant difference $P<0.01$ by ANOVA, respectively. When ANOVA was significant, post-hoc analyses were conducted using Tukey-Kramer multiple comparison test, with the level of statistical significance taken as $P<0.01$. Different letters adjacent to the symbol indicate the significant difference at each day at $P<0.01$ by Tukey-Kramer multiple comparison test.

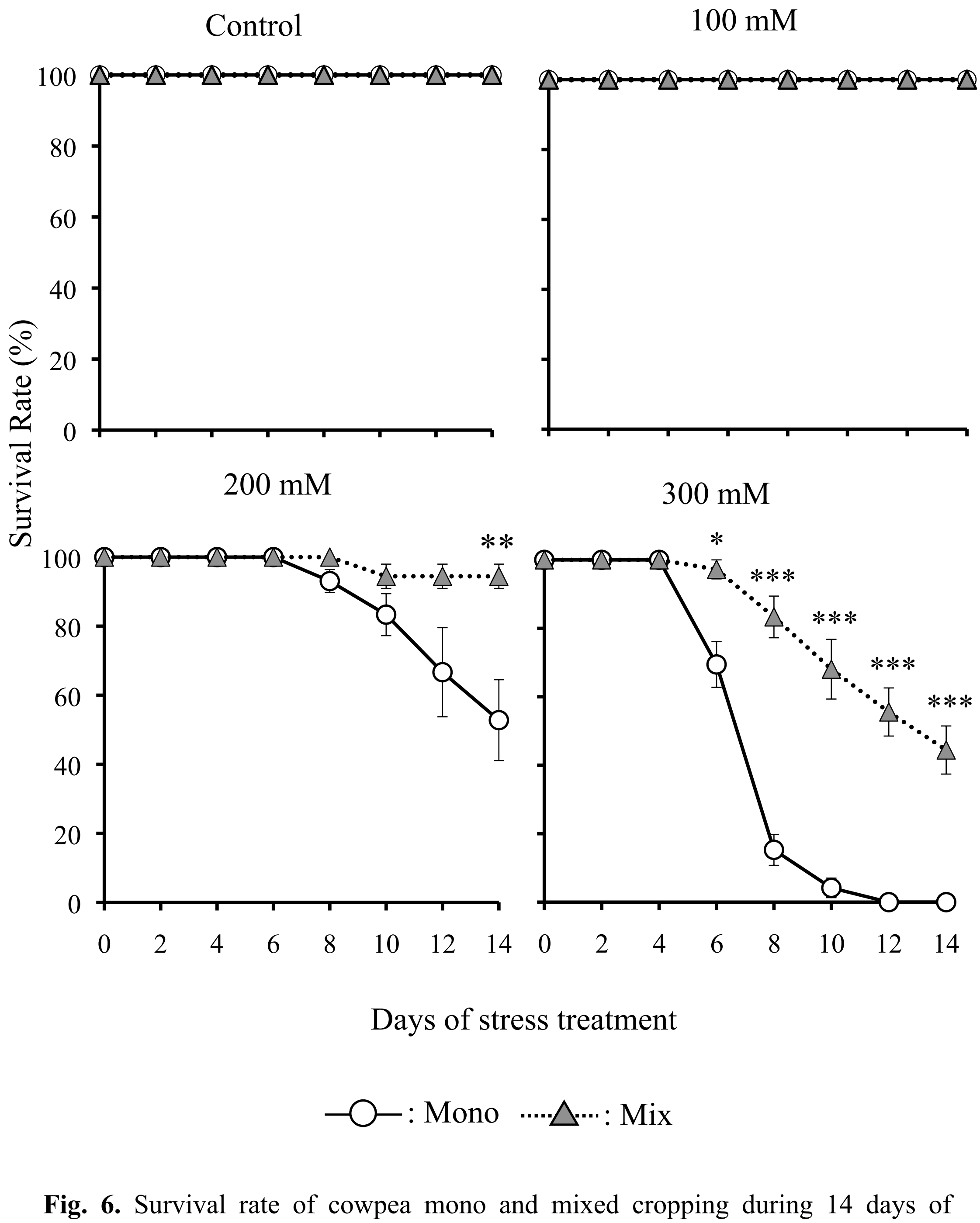

Fig. 6. Survival rate of cowpea mono and mixed cropping during 14 days of treatment with 100, 200 and 300 mM NaCl. The data are means ± SE (n = 6). The symbols of *, ** and *** indicate the significant difference between mono crop and mixed-crop at $P < 0.05$, 0.01 and 0.001 by independent samples *t*-test, respectively.

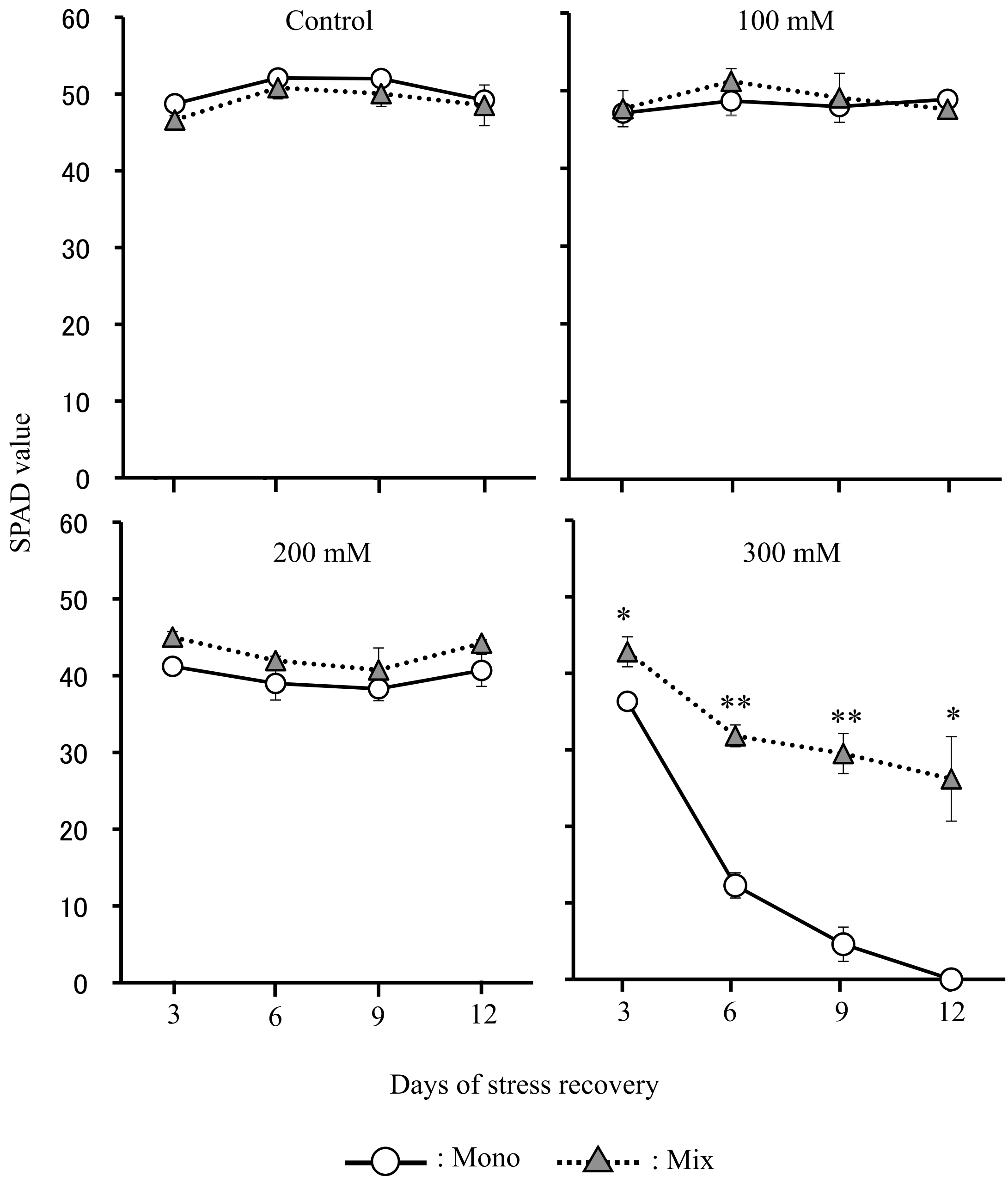

Fig. 7. Time course of the SPAD value of cowpea mono and mixed cropping after 3, 6, 9 and 12 days of recovery. Cowpea plants were treated with 100, 200 and 300 mM NaCl for three days, and then they were grown under the nutrient solution without NaCl for 14 days. Each value is the mean ± SE (n = 3). The symbols of * and ** indicate the significant difference between mono crop and mixed-crop at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

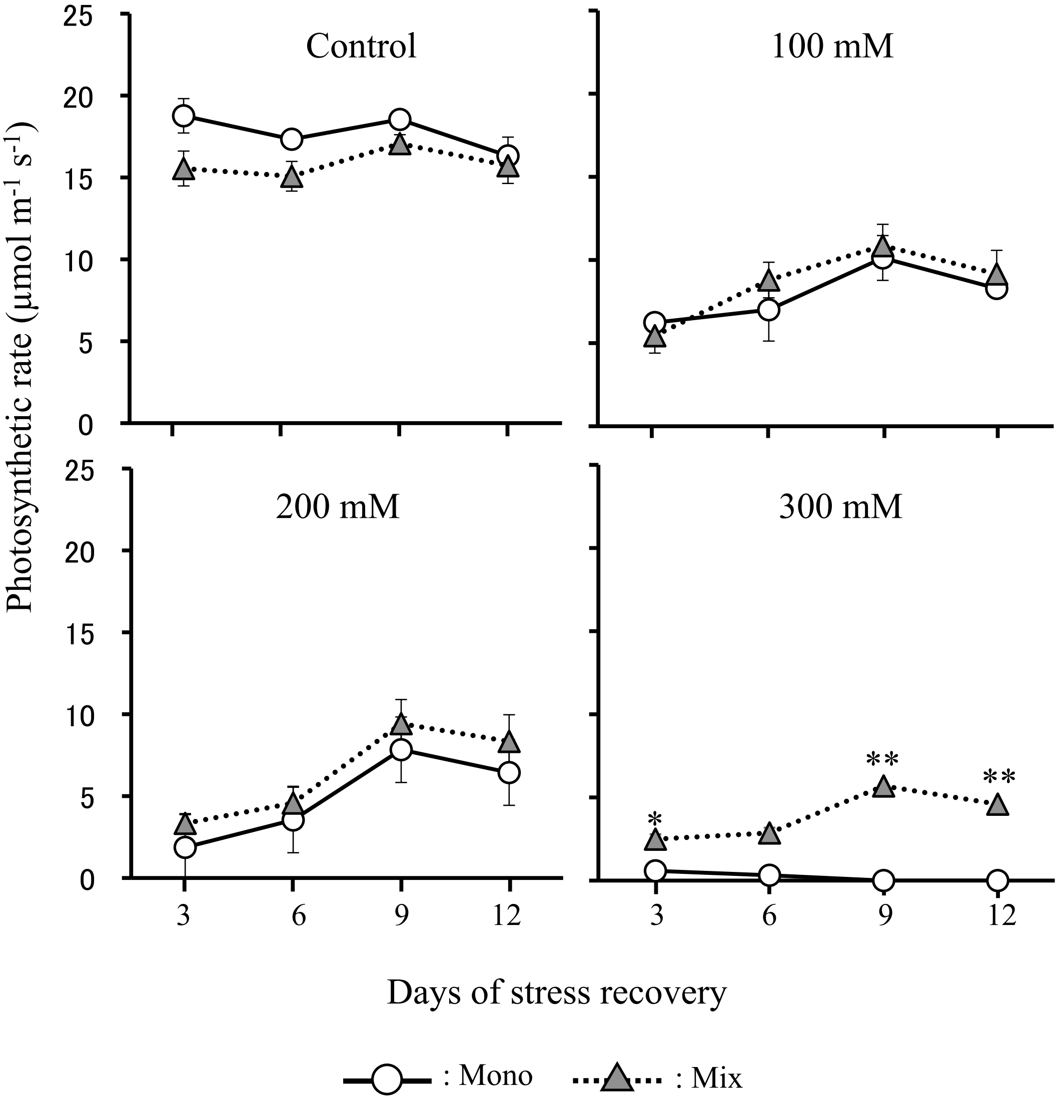

Fig. 8. Time course of the photosynthetic rate of cowpea mono and mixed cropping after 3, 6, 9 and 12 days of the recovery. Cowpea plants were treated with NaCl for three days, and then they were grown under the nutrient solution without NaCl for 14 days. Each value is the mean ± SE (n = 3). The symbols of * and ** indicate the significant difference between mono crop and mixed-crop at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

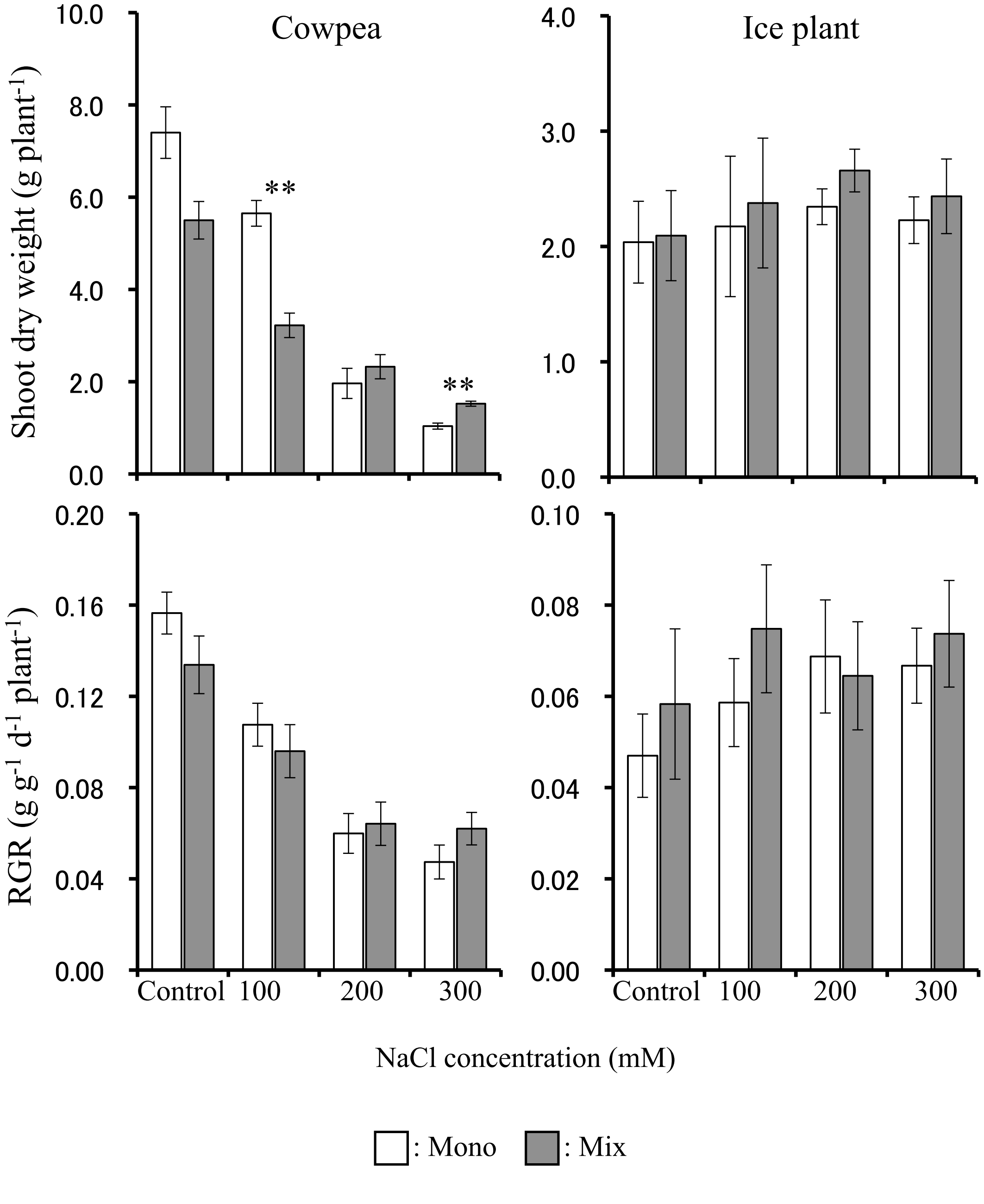

Fig. 9. Shoot dry weight and relative growth rate (RGR) of cowpea and ice plant of mono and mixed cropping at 14 days recovery after the treatment with 100, 200 and 300 mM NaCl for 3 days. The data are means ± SE (n = 3). The symbol of ** indicates the significant difference between mono crop and mixed-crop in each treatment at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

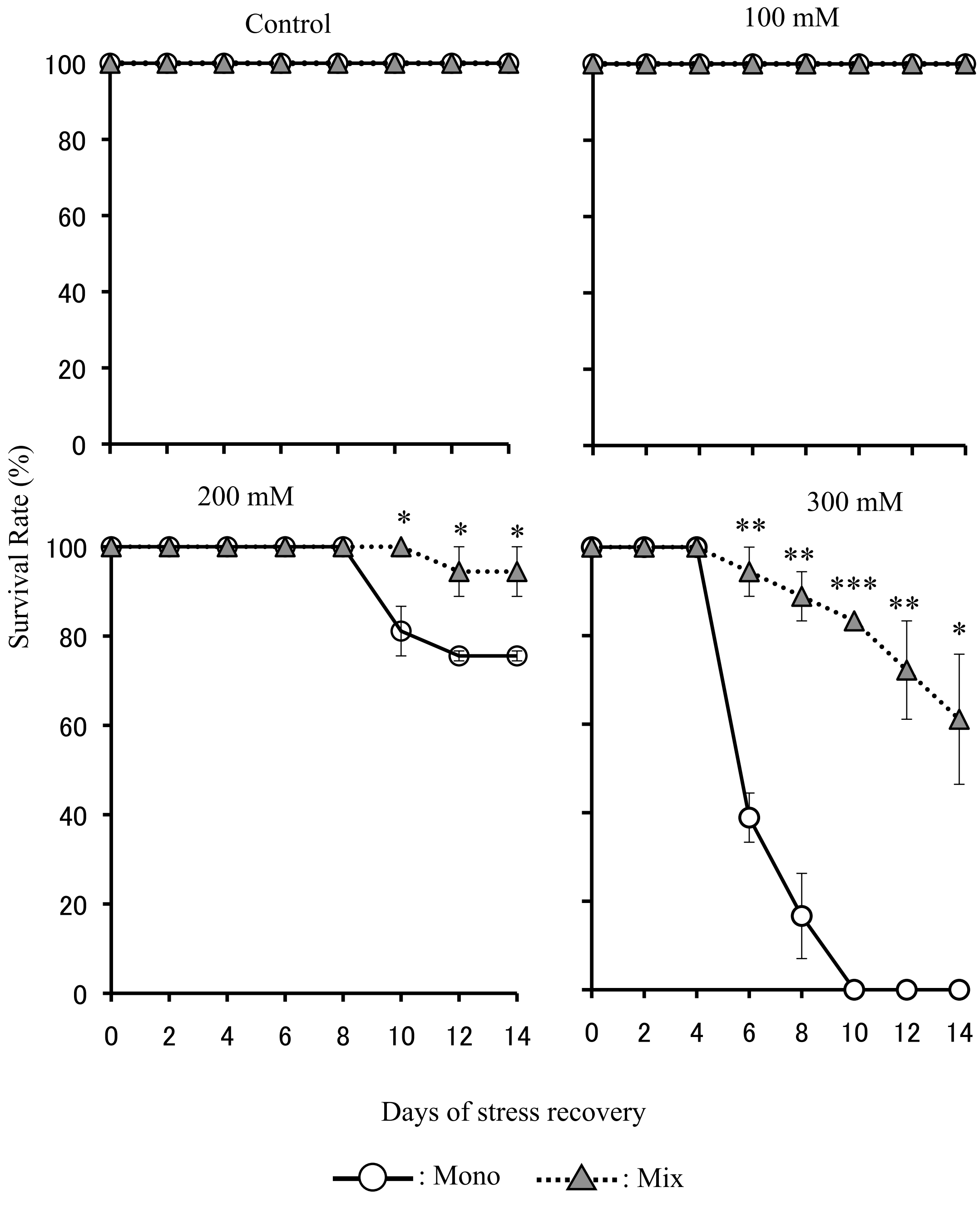

Fig. 10. Survival rate of cowpea mono and mixed cropping during 14 days of recovery from 3 days of treatment with 100, 200 and 300 mM NaCl followed. The data are means ± SE (n = 3). The symbols of *, ** and *** indicate the significant difference between mono crop and mixed-crop at $P < 0.05$, 0.01 and 0.001 by independent samples t-test, respectively.

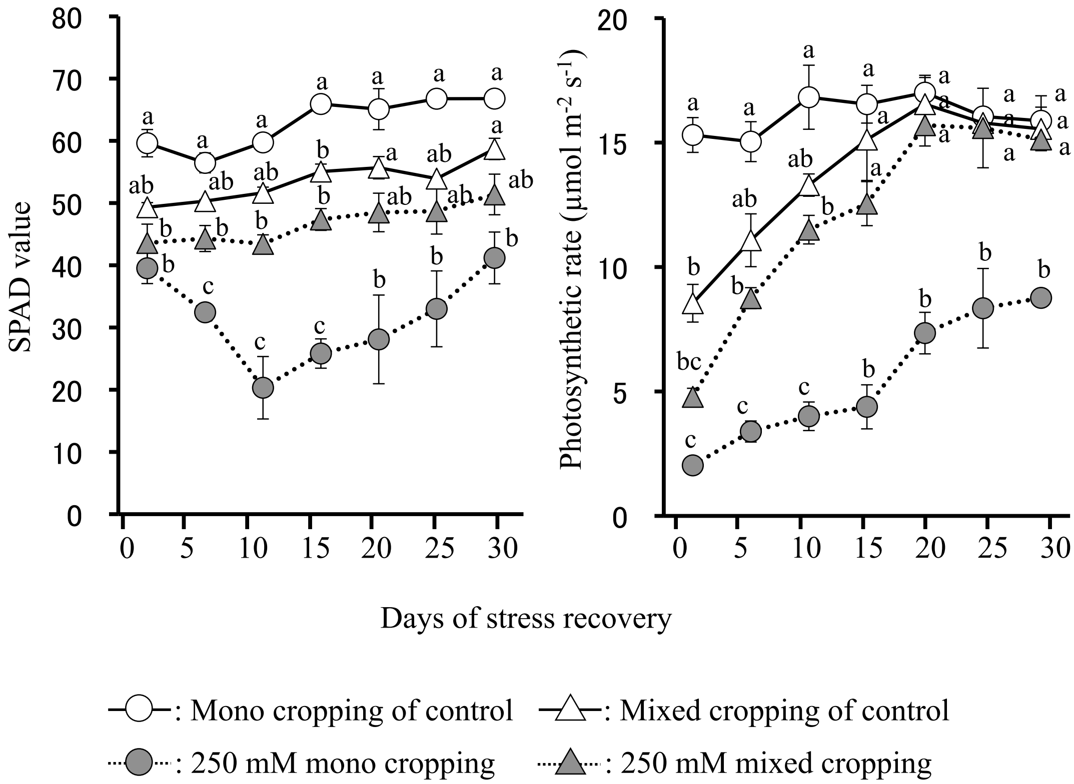

Fig. 11. Time course of the SPAD value (left) and the photosynthetic rate (right) of cowpea mono and mixed cropping during the recovery. Cowpea plants were treated with 250 mM NaCl for three days, and then they were transferred to 1/5000 a Wagner pot including soil for the recovery. Each value is the mean ± SE (n = 3). The different letters adjacent to the symbol indicate the significant difference at each day at $P < 0.01$ by Tukey's multiple comparison test.

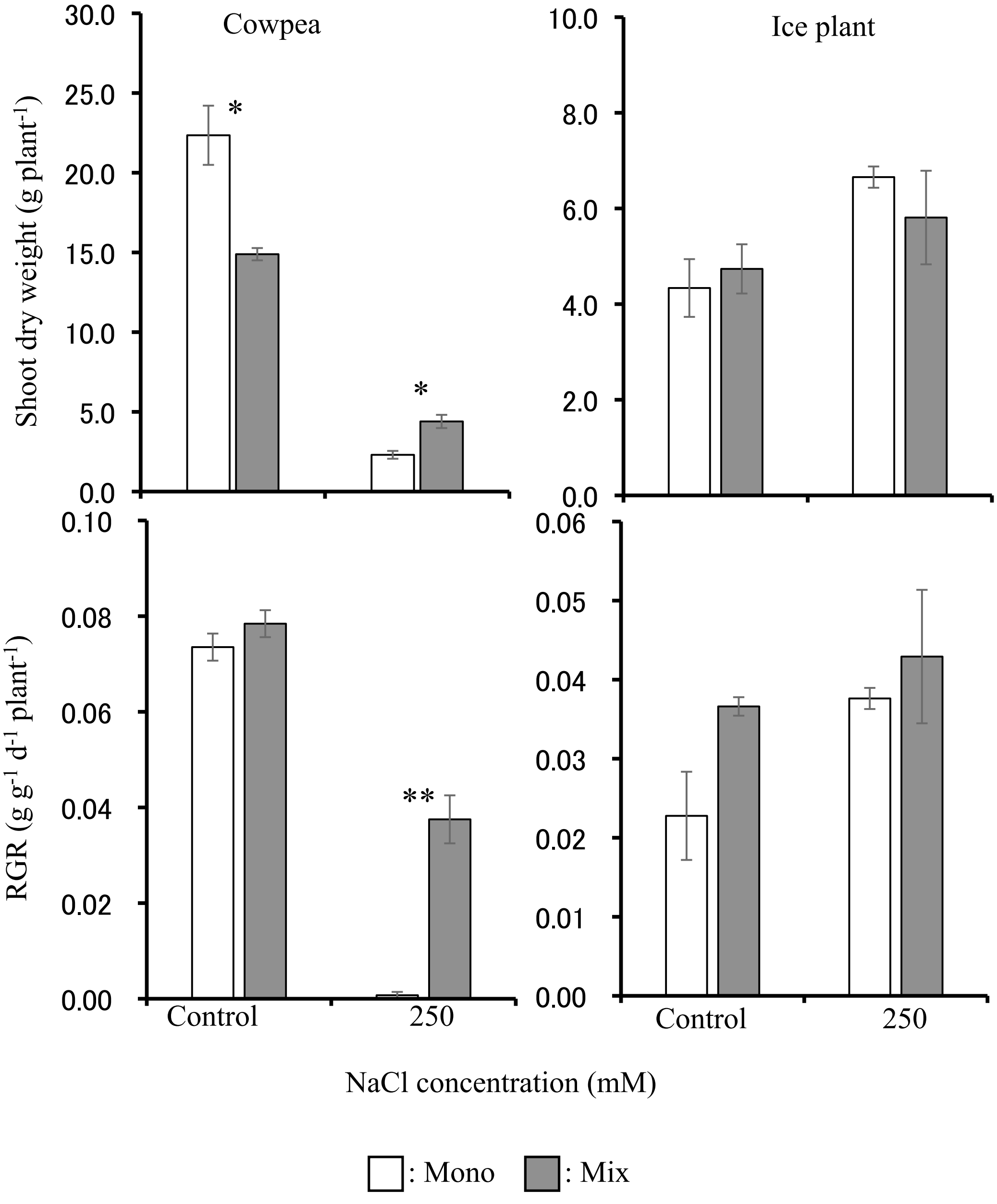

Fig. 12. Shoot dry weight and relative growth rate (RGR) of cowpea and ice plant mono and mixed cropping at 1 month recovery after the treatment with 250 mM NaCl for 3 days. The data are means ± SE (n = 3). The symbol of * and ** indicates the significant difference between mono crop and mix cropping at $P < 0.05$ and $P < 0.01$ by Student's t-test, respectively.

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