Phyton-International Journal of Experimental Botany
DOI:10.32604/phyton.2020.010160
Article

Differential Effects of Ammonium and Nitrate on Growth Performance of *Glechoma longituba* under Heterogeneous Cd Stress

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Received: 14 February 2020; Accepted: 27 March 2020

Abstract: Water, minerals, nutrients, etc., can be shared by physiological integration among inter-connected ramets of clonal plants. Nitrogen plays an important role in alleviating cadmium (Cd) stress for clonal plants. But how different forms of nitrogen affect growth performance of clonal plants subjected to heterogeneous Cd stress still remains poorly understood. A pot experiment was conducted to investigate the differential effects of ammonium and nitrate on growth performance of *Glechoma longituba* under heterogeneous Cd stress. In the experiment, parent ramets of *Glechoma longituba* clonal fragments were respectively supplied with modified Hoagland solution containing 7.5 mM ammonium, 7.5 mM nitrate or the same volume of nutrient solution without nitrogen. Cd solution with different concentrations (0, 0.1 or 2.0 mM) was applied to offspring ramets of the clonal fragments. Compared with control (N-free), nitrogen addition to parent ramets, especially ammonium, significantly improved antioxidant capacity [glutathione (GSH), proline (Pro), peroxidase (POD,) superoxide dismutase (SOD) and catalase (CAT)], PSII activity [maximum quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII (ΦPSII)], chlorophyll content and biomass accumulation of the offspring ramets suffering from Cd stress. In addition, negative effects of nitrate on growth performance of whole clonal fragments were observed under Cd stress with high concentration (2.0 mM). Transportation or sharing of nitrogen, especially ammonium, can improve growth performance of clonal plants under heterogeneous Cd stress. The experiment provides insight into transmission mechanism of nitrogen among ramets of clonal plants suffering from heterogeneous nutrient supply. Physiological integration might be an important ecological strategy for clonal plants adapting to heterogeneous environment stress conditions.

Keywords: Clonal plant; physiological integration; nitrogen form; antioxidant capacity; chlorophyll fluorescence

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1 Introduction

One of the most striking attributes of clonal plants is their capacity for physiological integration, which enables the inter-connected ramets to share water, carbohydrates, minerals and nutrients as well as signal substance [1–4]. Resources sharing among inter-connected ramets could buffer the negative influences of heterogeneous distribution of resources [2,5–7] and heterogeneous environment stress, such as wind erosion [8], water stress [6,9] and heavy metals stress [10].

Cadmium (Cd) is an extremely harmful pollutant in the environment, which seriously affects a variety of physiological processes of plants [11–13]. Proline content and antioxidant enzymes (peroxidase [POD] superoxide dismutase [SOD] and catalase [CAT]) play very important roles in maintaining redox homeostasis [14,15] and scavenging free radicals [16,17] and ROS for plants [13,18–19]. Cd stress generally induces nitrogen and carbon metabolism changes [11,20,21], water and mineral nutrition malabsorption [22,23], enzyme activities inhibition [21,24], reactive oxygen species (ROS) accumulation [24,25] and membrane damage [11,26]. Cd disrupts cellular redox homeostasis and generates the overproduction of reactive oxygen species (ROS) such as superoxide anion (O_{2}^{-}), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) [13,24,27]. Overproduction of ROS causes lipid peroxidation, electrolyte leakage, DNA and proteins damages in plants [28,29]. The phytotoxicities of Cd stress may result in poor growth, low biomass accumulation and death of plants [20,21].

Nitrogen addition may alleviate Cd stress for plants [30,31]. Ammonium (NH_4^+) and nitrate (NO_3^-) ions are the two major forms of nitrogen taken up by the root of plant. Ammonium is usually incorporated into amino acids (glutamine/glutamate) by the GS/GOGAT pathway [6,32]. Meanwhile, nitrate reduced and stored in the vacuoles. In addition, it is translocated to the shoot for reduction and vacuolar storage [32]. Compared with nitrate, ammonium is more effective on improving foliar water content, transpiration rate, stomatal conductance and photosynthetic activity of plants suffering from Cd stress [20,21,33]. When ammonium is taken up by plants, rhizosphere soil pH is decreased [33,34]. Meanwhile, ammonium competes with Cd^{2+} for absorption sites in roots [35–37], depolarizes membrane potential of root cells [33,38]. The decrease of rhizosphere soil pH enhances the absorption of Cd^{2+} [33,34], but the competitive absorption of ammonium and Cd^{2+} may inhibits Cd^{2+} uptake in plants as well as the change of membrane [33–37]. Conversely, when nitrate is taken up by plants, rhizosphere soil pH is increased, which diminishes the absorption of Cd^{2+} [33,39]. As a signal substance in plants, nitrate can up-regulate IRT_1 expression, facilitating the uptake of metal cation including Cd^{2+} [12,39]. How ammonium and nitrate application affect growth performance of clonal plants subjected to heterogeneous Cd stress still remains poorly understood.

A pot experiment was conducted by using *Glechoma longituba* clonal fragments under heterogeneous Cd stress. Each clonal fragment selected in the experiment consisted of parent and offspring ramets. The offspring ramets were treated with Cd solution with different concentrations (0, 0.1 and 2.0 mM), whereas the parent ramets were supplied with modified Hoagland solution containing ammonium or nitrate as the sole nitrogen source. In the control, parent ramets were supplied with the same volume of nutrient solution without nitrogen. Stolon between two successive ramets remained intact. In the experiment, we tested the following specific hypotheses. (1) The negative effects of Cd stress on growth performance of offspring ramets will be alleviated by the transportation or sharing of nitrogen from their connected parent ramets. (2) Ammonium will be more effective in buffering the negative effects of Cd stress on growth performance of offspring ramets than nitrate.

2 Materials and Methods

2.1 Plant Material and Treatment

Glechoma longituba (Nakai) Kupr is a stoloniferous perennial herb. A genet or fragment consists of a number of ramets connected by stolons. Ramets can develop on all stolon nodes. Each ramet has two

opposite single leaves originating from a stolon node. Every leaf axil bears one bud that may grow into a secondary stolon. The plant is generally found in forests, on roadsides or close to creeks and is distributed all over China except for the Northwest [40].

On June 15th 2015, clonal fragments of *Glechoma longituba* were selected from the plant stock with uniform size. Each fragment consisted of parent and offspring ramets. Parent and offspring ramets of each pair were rooted individually in 0.5 L plastic pots filled with acid washed sand. The experimental design consisted of two crossed factors: 'nitrogen addition' (N-free, ammonium and nitrate) and 'Cd stress' (0, 0.1 and 2.0 mM Cd). Parent ramets were supplied with 50 mL modified Hoagland solution containing 7.5 mM ammonium or 7.5 mM nitrate as the sole nitrogen source (Tab. 1). Meanwhile, 50 mL Cd solution with different concentration (0, 0.1 and 2.0 mM) was applied to offspring ramets. Stolon between two successive ramets remained intact (Please see Fig. 1). Modified Hoagland solution and Cd solution were applied to parent and offspring ramets respectively every three days. To avoid nutrient or Cd build-up, all pots were watered every two days [6]. The experiment was performed in a greenhouse for 45 days. During the experiment, the mean temperature in the glasshouse was 28.5 ± 1.4°C and the relative humidity 70.5 ± 2.5%. The minimum and maximum phototsynthetic photon flux density (PPFD) in the glasshouse were 136.2 and 325.1 μmol m^{-2} s^{-1}, respectively. Each treatment was replicated 8 times. No ramet died during the experiment.

Table 1: The composition of modified Hoagland solution containing different forms of nitrogen or without nitrogen

Nutrient composition	Ammonium treatment (μM)	Nitrate treatment (μM)	N-free (μM)
$(NH_4)_2SO_4$	3,750	0	0
KNO_3	0	7,500	0
K_2SO_4	3,750	0	3,750
Na_2SO_4	0	7,500	3,750
NaH_2PO_4	3,750	3,750	3,750
$MgSO_4$	2,500	2,500	2,500
$CaCl_2$	5,000	5,000	5,000
H_3BO_3	50	50	50
$MnSO_4$	2.5	2.5	2.5
$ZnSO_4$	2.5	2.5	2.5
$CuSO_4$	0.5	0.5	0.5
Na_2MoO_4	1	1	1
Fe-EDTA	100	100	100

2.2 Determination of GSH and Pro Contents

Total glutathione (GSH) content was measured according to the spectrophotometric method [41,42]. This method is based on the reduction of oxidized glutathione (GSSG) to reduced GSH using the GSH-reductase recycling procedure. With 50 $g \cdot L^{-1}$ tri-chloroacetic acid (TCA), 1.0 g fresh leaves of offspring ramets were ground and centrifuged at 12,000 × *g* for 20 min. The supernatant was used for GSH assay. In the presence of glutathione reductase (GR) and NADPH, total GSH (GSH + GSSG) was determined by the colorimetric reaction of DTNB [5, 5'-dithio-bis (2-nitrobenzoic acid)] with GSH to form TNB

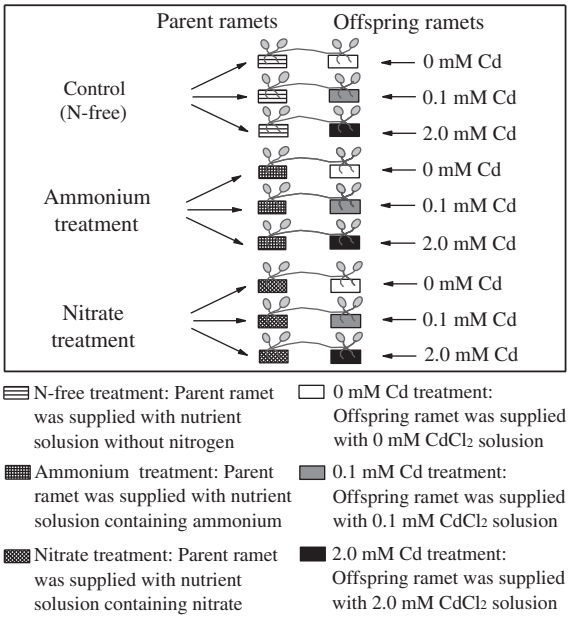

Figure 1: Schematic representation of the experimental design by using *Glechoma longituba* clonal fragments

(5-thio-2 nitrobenzoic acid). The rate of TNB formation, which was proportional to the total GSH concentration, was measured spectrophotometrically at 412 nm.

Proline was determined according to the procedure described by Bates et al. [43]. Fresh leaves (0.5 g) of offspring ramets were used to extract Pro with 3% aqueous 5-sulphosalicylic acid, centrifuged at 10,000 × *g*. The sample of the supernatant was used for the Pro assay and measured at 520 nm.

2.3 Antioxidant Enzymes Extraction and Assays

Two days before harvest, in a mortar, 0.5 g fresh leaves of offspring ramets were ground to a fine powder with liquid nitrogen. Then, the fine powder was extracted in 3 ml of 50 mM sodium phosphate buffer (pH 7.8) including 1.0 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 10000 × *g* for 30 min and the supernatant was stored at −75°C for SOD, POD and CAT activity analyses.

SOD activity was determined according to the method of Beauchamp et al. [44]. The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin and 0.2 mL of the enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes under two 20 W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. The photoreduction of NBT (production of blue formazan) was measured at A_{560}. One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as SOD units per g of fresh plant weight.

POD activity was determined according to the method of Zhang et al. [13]. In the presence of H_2O_2, POD catalyzes the transformation of guaiacol to tetraguaiacol (brown product). The oxidation of guaiacol was measured by the increase in absorbance at 470 nm for 1 min (ε = 26.6 L·mmol^{-1} cm^{-1}). The reaction mixture contained 50 μL of 20 mM guaiacol, 2.7 mL of 10 mM phosphate buffer (pH 7.0), and 0.2 mL enzyme extract. The reaction was started with 20 μL of 40 mM H_2O_2. One unit of POD activity was expressed as POD units per min and g of fresh plant weight.

CAT activity was measured according the method of Aebi [45], with minor modifications. The reaction mixture (1.5 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 20 mM H_2O_2 and 50 μL enzyme extract. The reaction was started by addition of the extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 $L \cdot mmol^{-1}$ cm^{-1}) and the results expressed as CAT units per min and g of fresh plant weight.

2.4 Measurement of Chlorophyll Fluorescence Parameters and Chlorophyll Content

Chlorophyll fluorescence parameters were measured by the saturation pulse method [6,46] with a portable chlorophyll fluorometer (PAM-2100, Walz, Effeltrich, Germany). A pulse of saturating light (>4000 μmol photons m^{-2} s^{-1}, 0.8 s pulse length, actinic white light) was applied through an optical fibre at an angle of 60° relative to the sample and a distance of 12 mm from the leaf. Measurements were made on the upper surface of the two leaves of each original offspring ramet two days before harvest. The maximum quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII (ΦPSII) were determined. We calculated the maximum quantum yield of PSII as the ratio $F_v/F_m = (F_m - F_0)/F_m$ [47], where F_0 and F_m are the minimal and maximal fluorescence yields of a dark-adapted sample, respectively, with all PSII reaction centers fully open (i.e., all primary acceptors oxidized). These parameters were measured after dark adaptation for 30 min. The F_v/F_m ratio provides an estimate of the efficiency of excitation energy capture by open PSII reaction centers [48]. We calculated ΦPSII as $(F'_m - F_t)/F'_m$ [49], where F'_m is the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample and Ft is the fluorescence yield of the leaf at a given photosynthetic photon flux density. ΦPSII is a measure of the fraction of the light absorbed by chlorophyll that is photochemically converted into PSII [50].

Total chlorophyll was extracted from 0.5 g fresh leaves of offspring ramets using 80% (v/v) acetone. Absorbance was measured at 663 and 645 nm by a spectrophotometer (UV-2450, Shimadiu Corporation, Japan). Extinction coefficients and equations reported by [51] were used to calculate the amounts of total chlorophyll content.

2.5 Determination of Biomass Accumulation

At the end of the experiment, parent and offspring ramets were harvested separately and dried to constant weight at 70°C to determine dry mass. The newly produced stolons and new ramets were assigned to the ramets (initial parent or offspring ramets) producing them.

2.6 Statistical Analyses

Prior to analysis, Pro content, SOD activity and POD activity were square root-transformed to meet the requirements of normality and homoscedasticity for ANOVA. Differences among treatments for antioxidant capacity, PSII activity, total chlorophyll content and biomass accumulation were evaluated by two-way ANOVA with ‘nitrogen addition’ and ‘Cd stress’ as the main factors. Fisher’s least-significant difference test (LSD) was employed to compare the means in all measured characters (SPSS 23.0).

3 Results

3.1 Antioxidant Capacity of Offspring Ramets

Foliar antioxidant enzyme (SOD, POD and CAT) activities of offspring ramets were significantly affected by nitrogen addition, Cd stress and their interaction as well as contents of GSH and Pro (Tab. 2). Compared with control, ammonium addition to parent ramets significantly increased foliar GSH content, SOD, POD and CAT activity by 14.9%, 11.9%, 22.0% and 257.5% in offspring ramets subjected to low concentration (0.1 mM) Cd stress (Figs. 2A, 2C–2E). Nitrate addition to parent ramets significantly increased foliar Pro content, SOD, POD and CAT activity by 85.0%, 5.1%, 27.4% and 46.9% in offspring ramets subjected to low concentration (0.1 mM) Cd stress (Figs. 2B–2E). But it significantly reduced foliar GSH content of offspring ramets (Fig. 2A). Compared with nitrate, GSH content SOD and CAT

Table 2: Effects of nitrogen addition, Cd stress and their interaction on antioxidant capacity of offspring ramets

Effect	df		Foliar GSH content	Foliar Pro content	Foliar SOD activity	Foliar POD activity	Foliar CAT activity
Nitrogen addition (N)	2	*F*	1725.444	279.269	1065.097	160.063	1027.349
		P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Cadmium stress (Cd)	2	*F*	6987.554	739.841	733.385	841.315	395.711
		P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
N × Cd	4	*F*	1102.832	229.241	47.994	154.181	257.841
		P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Error	63						
Total	71						

Values are in bold where $P < 0.05$.

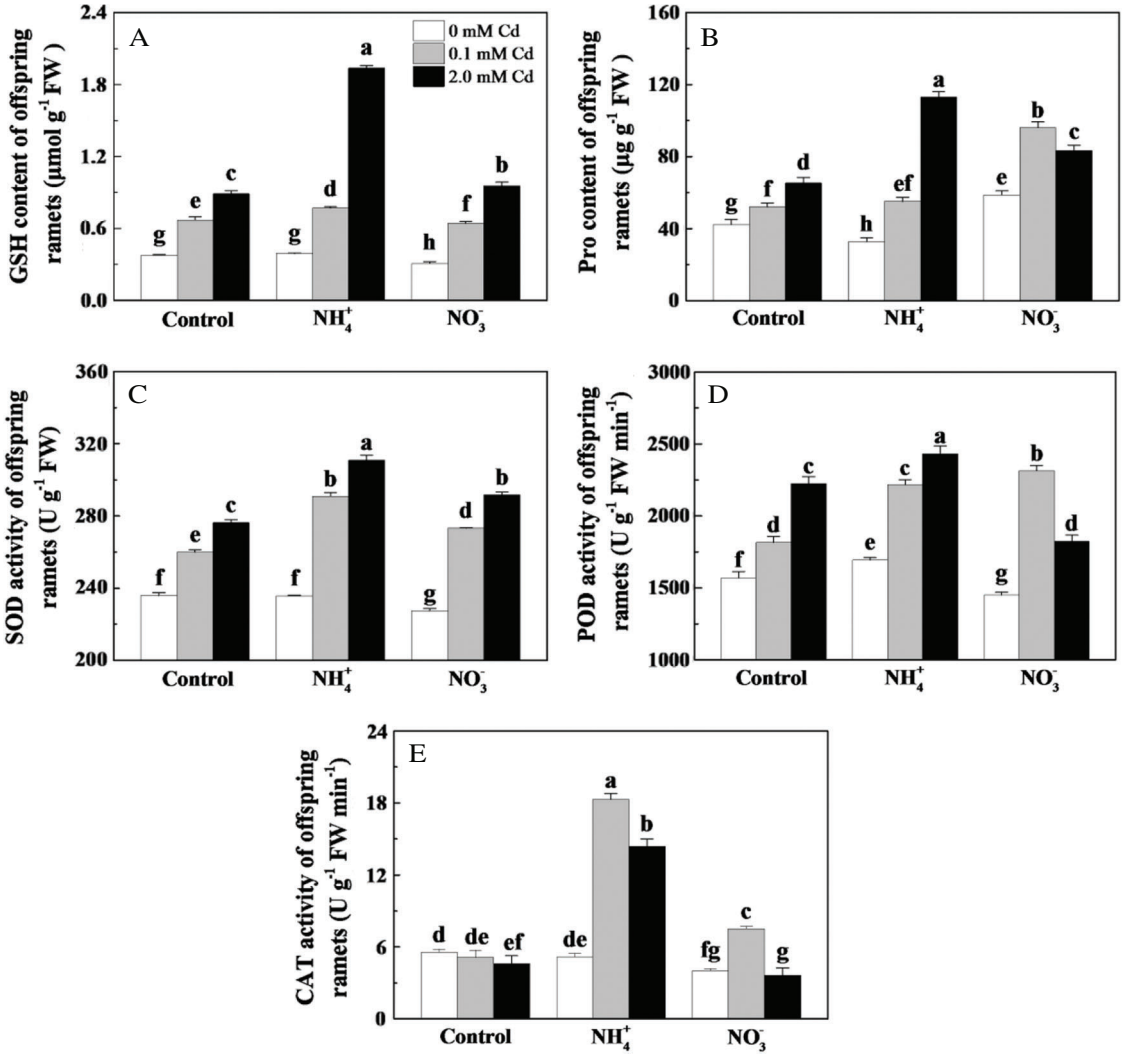

Figure 2: Foliar GSH content (A), Pro content (B), SOD activity (C), POD activity (D) and CAT activity (E) of offspring ramets suffering from different concentrations (0, 0.1 and 2.0 mM) of Cd stress in the control and nitrogen (ammonium and nitrate) addition treatments. Bars marked with different lowercase letters are significantly different at $p \leq 0.05$

activity of offspring ramets in ammonium treatments were significantly higher by 14.9%, 11.9% and 143.5%, respectively (Figs. 2A, 2C, 2E); but Pro content and POD activity of offspring ramets were lower, respectively by 84.3% and 4.4% (Figs. 2B, 2D).

Under high concentration (2.0 mM) Cd stress, foliar GSH and Pro contents, SOD, POD and CAT activities of offspring ramets were increased, respectively by 117.9% 73.1%, 12.5%, 9.3% and 210.4% in ammonium addition as compared to control (Figs. 2A–2E). In nitrate treatment, foliar GSH and Pro contents, and SOD activity of offspring ramets were significantly increased, respectively by 7.6%, 27.5% and 5.6% as compared to control (Figs. 2A–2C). However, foliar POD and CAT activities of offspring ramets was significantly decreased 21.9% and 26.8% in nitrate treatment (Figs. 2D, 2E). Compared with nitrate, GSH and Pro contents, SOD, POD and CAT activities were higher, respectively by 45.8%, 35.8%, 6.5%, 33% and 294% (Figs. 2A–2E).

3.2 PSII Activity and Total Chlorophyll Content of Offspring Ramets

Foliar PSII activity (F_v/F_m and ΦPSII) and total chlorophyll content of offspring ramets were significantly affected by nitrogen addition, Cd stress and their interaction (Tab. 3). Compared with control, foliar F_v/F_m, ΦPSII and total chlorophyll content of offspring ramets subjected to low concentration (0.1 mM) Cd stress were significantly increased, respectively by 14.3%, 43.7% and 66.7% when ammonium was added to parent ramets; Nitrate addition to parent ramets significantly increased foliar F_v/F_m, and ΦPSII of offspring ramets suffering from low concentration (0.1 mM) Cd stress, respectively by 5.9% and 18.7%. Meanwhile, F_v/F_m, ΦPSII and total chlorophyll content of offspring ramets in ammonium treatments were 7.5%, 21.1% and 58.3% higher, respectively, than those in nitrate treatment (Figs. 3A–3C).

Table 3: Effects of nitrogen addition, Cd stress and their interaction on PSII activity and total chlorophyll content of offspring ramets

Effect	df		F_v/F_m	ΦPSII	Total chlorophyll content
Nitrogen addition (N)	2	*F*	35.977	19.610	251.770
		p	**<0.001**	**<0.001**	**<0.001**
Cadmium stress (Cd)	2	*F*	161.531	56.779	107.005
		p	**<0.001**	**<0.001**	**<0.001**
N × Cd	4	*F*	10.391	3.240	40.698
		p	**<0.001**	**0.020**	**<0.001**
Error	63				
Total	71				

Values are in bold where $P < 0.05$.

When offspring ramets subjected to high concentration (2.0 mM) Cd stress, ammonium addition to parent ramets significantly increased foliar F_v/F_m, ΦPSII and total chlorophyll content of offspring ramets, respectively by 11.5%, 29% and 22.6%, as compared with control (Figs. 3A–3C); however, similar patterns were not observed in nitrate treatment (Figs. 3A–3C). Compared with nitrate, foliar F_v/F_m, ΦPSII and total chlorophyll content of offspring ramets in ammonium treatments were significantly higher by 15.3%, 29% and 30%, respectively (Figs. 3A–3C).

3.3 Biomass Accumulation of Clonal Fragments

Biomass accumulation of clonal fragments were significantly affected by nitrogen addition and Cd stress as well as parent and offspring ramets. Meanwhile, significant interaction of nitrogen addition and Cd stress on biomass accumulation of offspring ramets and clonal fragments was detected (Tab. 4). Compared with

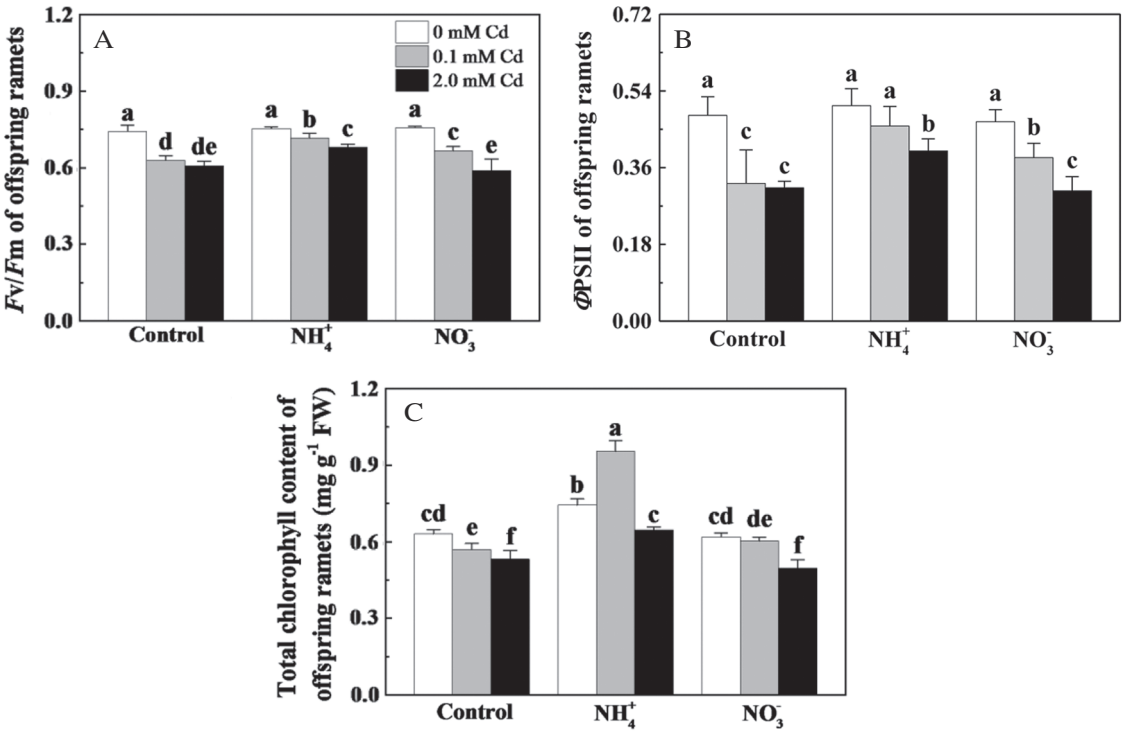

Figure 3: Foliar F_v/F_m (A), ΦPSII (B) and total chlorophyll content (C) of offspring ramets suffering from different concentrations (0, 0.1 and 2.0 mM) of Cd stress in the control and nitrogen (ammonium and nitrate) addition treatments. Bars marked with different lowercase letters are significantly different at $p \leq 0.05$

Table 4: Effects of nitrogen addition, Cd stress and their interaction on biomass accumulation of parent ramets, offspring ramets and clonal fragment

Effect	df		Parent ramets	Offspring ramets	Clonal fragment
Nitrogen addition (N)	2	*F*	53.373	**33.624**	**175.343**
		p	**<0.001**	**<0.001**	**<0.001**
Cadmium stress (Cd)	2	*F*	32.002	**90.518**	**248.483**
		p	**<0.001**	**<0.001**	**<0.001**
N × Cd	4	*F*	2.384	**9.843**	**22.613**
		p	0.076	**<0.001**	**<0.001**
Error	63				
Total	71				

Values are in bold where $P < 0.05$.

control, biomass accumulation of clonal fragments, parent and offspring ramets were significantly increased 71.8%, 66.6% and 77.9% in ammonium and 41.4% 50.7% and 28.8% in nitrate treatments, respectively, when offspring ramets suffering from low concentration Cd stress (0.1 mM) (Fig. 4). Biomass accumulation of clonal fragments and offspring ramets in ammonium treatments were 21.5% and 38.1% higher, respectively, than those in nitrate treatment.

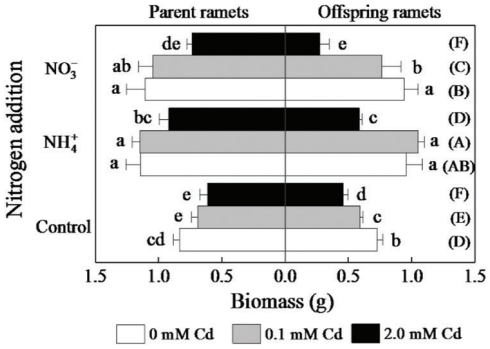

Figure 4: Biomass accumulation of parent, offspring ramets and clonal fragments in the control and nitrogen (ammonium and nitrate) addition treatments. The offspring ramets suffered from different concentrations (0, 0.1 and 2.0 mM) of Cd stress. Bars marked with different letters are significantly different at $p \leq 0.05$. Lower case letters represent parent and offspring ramets, and upper case letters represent whole clonal fragments

When offspring ramets suffering from high concentration Cd stress (2 mM), compared with control, biomass accumulation of clonal fragments, parent and offspring ramets were significantly increased 38.9%, 50.8% and 26.1% in ammonium treatments; however, biomass accumulation of offspring ramets was significantly decreased 70.2% in nitrate treatment. Compared with nitrate, biomass accumulation of clonal fragments, parent and offspring ramets in ammonium treatments were significantly higher by 48.5%, 24.3% and 114.8%, respectively.

4 Discussion

Our results supported the first hypothesis that negative effects of Cd stress on growth performance of offspring ramets could be alleviated by transportation or sharing of nitrogen from their connected parent ones. Compared with nitrate, ammonium addition to parent ramets significantly improved foliar antioxidant capacity and PSII activity of offspring ramets suffering from Cd stress as well as greater total chlorophyll content and biomass accumulation. It was concluded that ammonium could be more effective in buffering negative effects of Cd stress on growth performance of offspring ramets than nitrate. So, our results further supported the second hypothesis.

Pro, GSH and antioxidant enzymes may play an important role in alleviating Cd stress to plants [24,51,52]. Meanwhile, synthesis of GSH, Pro and antioxidant enzymes requires the participation of nitrogen [31]. Transportation or sharing of nitrogen, especially ammonium, incurred a significant increase of foliar GSH and Pro content in offspring ramets suffering from Cd stress as well as enhancement of SOD, POD and CAT activities, thereby improving their resistance to Cd stress in the experiment. This result was consistent with previous finding [20].

Cd stress causes structural changes in chloroplasts [21,53], reduces chlorophyll synthesis [54–56] and inhibits photosynthetic enzymes activities [25]. Nitrogen addition to parent ramets, especially ammonium, significantly relieved negative effects of Cd stress on PSII activity (F_v/F_m and ΦPSII) and chlorophyll synthesis in offspring ramets suffering from Cd stress. A possible explanation is that transportation or sharing of nitrogen immediate promotes chlorophyll synthesis of offspring ramets suffering from Cd stress [31]. Further, increase of chlorophyll content can improve foliar efficiency to harvest light and activity of photosystem II [57].

Compared to control, nitrate addition to parent ramets significantly reduced biomass accumulation of offspring ramets suffering from Cd stress with higher concentration (2 mM). Nitrate as a signal substance, can up-regulate IRT1 expression, thereby facilitating the uptake of Cd^{2+} and developing more severe

toxic symptoms in plants [12,39]. In addition, nitrate transport across the plasma membrane results in temporary depolarized of the membrane potential in plant cell [33,58,59]. The depolarized the membrane potential may enhance the membrane transport of Cd^{2+} in plant [33,58,59]. However, uptake of ammonium reduces the membrane potential, which inhibits Cd^{2+} uptake [33,60]. In the experiment, nitrate addition to parent ramets caused higher foliar Cd content in offspring ramets than control (N-free) or ammonium (data not shown).

Transportation or sharing of nitrogen among ramets increased growth of clonal plants suffering from heterogeneous nutrient supply [61,62]. Ammonium or nitrate could be transported from parent ramets to their connected offspring ones [6]. Under heterogeneous Cd stress, differential effects of ammonium or nitrate addition to parent ramets on growth performance of offspring ramets and whole clonal fragments were observed in the experiment. However, further studies are needed to explore transmission mechanism of different nitrogen forms among ramets of clonal plants.

Our experiment provides evidence that ammonium will be more effective than nitrate in buffering negative effects of heterogeneous Cd stress on clonal plants. We tentatively conclude that preferential transportation or sharing of ammonium among ramets could be advantageous for clonal plants adapting to heterogeneous Cd stress [6]. Further studies on a wider range of species are needed to understand the generality of this pattern and to assess fully the ecological advantages afforded by these features.

Funding Statement: This research was supported by Science and Technology Support Program of Sichuan Province (2016NYZ0021) and Guizhou Environmental Protection Department (2016).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Březina, S., Koubek, T., Münzbergová, Z., Herben, T. (2006). Ecological benefits of integration of *Calamagrostis epigejos* ramets under field conditions. *Flora, 201(6),* 461–467. DOI 10.1016/j.flora.2005.10.003.
2. Xiao, K. Y., Yu, D., Wang, L. G., Han, Y. Q. (2011). Physiological integration helps a clonal macrophyte spread into competitive environments and coexist with other species. *Aquatic Botany, 95(4),* 249–253. DOI 10.1016/j.aquabot.2011.07.002.
3. Wei, G. W., Shu, Q., Luo, F. L., Chen, Y. H., Dong, B. C. et al. (2018). Separating effects of clonal integration on plant growth during submergence and de-submergence. *Flora, 246,* 118–125. DOI 10.1016/j.flora.2018.08.004.
4. Zhang, L. M., Lu, H. Z., Alpert, P., Song, L., Liu, W. Y. et al. (2019). Higher benefits of clonal integration in rhizome-derived than in frond-derived ramets of the tropical fern *Bolbitis heteroclita*. *Flora, 257,* 151415. DOI 10.1016/j.flora.2019.06.001.
5. Du, J., Wang, N., Alpert, P., Yu, M. J., Yu, F. H. et al. (2010). Clonal integration increases performance of ramets of the fern *Diplopterygium glaucum* in an evergreen forest in southeastern China. *Flora, 205(6),* 399–403. DOI 10.1016/j.flora.2009.12.018.
6. Roiloa, S. R., Antelo, B., Retuerto, R. (2014). Physiological integration modifies $\delta^{15}N$ in the clonal plant *Fragaria vesca*, suggesting preferential transport of nitrogen to water-stressed offspring. *Annals of Botany, 114(2),* 399–411. DOI 10.1093/aob/mcu064.
7. Zhou, J., Li, H. L., Alpert, P., Zhang, M. X., Yu, F. H. (2017). Fragmentation of the invasive, clonal plant *Alternanthera philoxeroides* decreases its growth but not its competitive effect. *Flora, 228,* 17–23. DOI 10.1016/j.flora.2017.01.007.
8. Yu, F. H., Wang, N., He, W. M., Chu, Y., Dong, M. (2008). Adaptation of rhizome connections in drylands: Increasing tolerance of clones to wind erosion. *Annals of Botany, 102(4),* 571–577. DOI 10.1093/aob/mcn119.

9. Wei, Q., Li, Q., Jin, Y., Li, K. N., Lei, N. F. et al. (2019). Effects of clonal integration on photochemical activity and growth performance of stoloniferous herb *Centella asiatica* suffering from heterogeneous water availability. *Flora, 256,* 36–42. DOI 10.1016/j.flora.2019.05.001.
10. Xu, L., Wu, X., Zhou, Z. F. (2016). Effects of physiological integration and fertilization on heavy metal remediation in soil by a clonal grass. *Polish Journal of Environmental Studies, 25(1),* 395–404. DOI 10.15244/pjoes/60374.
11. Chaffei, C., Pageau, K., Suzuki, A., Gouia, H., Ghorbel, M. H. et al. (2004). Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. *Plant and Cell Physiology, 45(11),* 1681–1693. DOI 10.1093/pcp/pch192.
12. Luo, B. F., Du, S. T., Lu, K. X., Liu, W. J., Lin, X. Y. et al. (2012). Iron uptake system mediates nitrate-facilitated cadmium accumulation in tomato (*Solanum lycopersicum*) plants. *Journal of Experimental Botany, 63(80),* 3127–3136. DOI 10.1093/jxb/ers036.
13. Zhang, F. Q., Wang, Y. S., Lou, Z. P., Dong, J. D. (2007). Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). *Chemosphere, 67(1),* 44–50. DOI 10.1016/j.chemosphere.2006.10.007.
14. Chen, C., Wanduragala, S., Becker, D. F., Dickman, M. B. (2006). Tomato QM-like protein protects *Saccharomyces cerevisiae* cells against oxidative stress by regulating intracellular proline levels. *Applied and Environmental Microbiology, 72(6),* 4001–4006. DOI 10.1128/AEM.02428-05.
15. Hoque, M. A., Banu, M. N. A., Nakamura, Y., Shimoishi, Y., Murata, Y. (2007). Proline and glycinebetaine enhance antioxidant defense and methylglyoxal detoxification systems and reduce NaCl-induced damage in cultured tobacco cells. *Journal of Plant Physiology, 165(8),* 813–824. DOI 10.1016/j.jplph.2007.07.013.
16. Hasegawa, P. M., Bressan, R. A., Zhu, J. K. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology, 51(1),* 463–499. DOI 10.1146/annurev.arplant.51.1.463.
17. Okuma, E., Soeda, K., Tada, M., Murata, Y. (2000). Exogenous proline mitigates the inhibition of growth of *Nicotiana tabacum* cultured cells under saline conditions. *Soil Science and Plant Nutrition, 46(1),* 257–263. DOI 10.1080/00380768.2000.10408781.
18. Dinakar, N., Nagajyothi, P. C., Suresh, S., Udaykiran, Y., Damodharam, T. (2008). Phytotoxicity of cadmium on protein, proline and antioxidant enzyme activities in growing *Arachis hypogaea* L. seedlings. *Journal of Environmental Sciences, 20(2),* 199–206. DOI 10.1016/S1001-0742(08)60032-7.
19. Islam, M. M., Hoque, A., Okuma, E., Nasrin, M., Banu, A. et al. (2009). Exogenous proline and glycinebetaine increase antioxidant enzyme activities and confer tolerance to cadmium stress in cultured tobacco cells. *Journal of Plant Physiology, 166(15),* 1587–1597. DOI 10.1016/j.jplph.2009.04.002.
20. Jalloh, M. A., Chen, J., Zhen, F., Zhang, G. (2009). Effect of different N fertilizer forms on antioxidant capacity and grain yield of rice growing under Cd stress. *Journal of Hazardous Materials, 162(2–3),* 1081–1085. DOI 10.1016/j.jhazmat.2008.05.146.
21. Nasraoui-Hajaji, A., Gharbi, F., Ghorbel, M. H., Gouia, H. (2010). Cadmium stress effects on photosynthesis and PSII efficiency in tomato grown on NO_3^- or NH_4^+ as nitrogen source. *Acta Botanica Gallica, 157(1),* 101–115. DOI 10.1080/12538078.2010.10516192.
22. Toppi, L. S. D., Gabbrielli, R. (1999). Response to cadmium in higher plants. *Environmental and Experimental Botany, 41(2),* 105–130. DOI 10.1016/S0098-8472(98)00058-6.
23. Parida, A. K., Das, A. B., Mittra, B. (2003). Effects of NaCl stress on the structure, pigment complex composition, and photosynthetic activity of mangrove *Bruguiera parviflora* chloroplasts. *Photosynthetica, 41(2),* 191–200. DOI 10.1023/B:PHOT.0000011951.37231.69.
24. Pereira, G. J. G., Molina, S. M. G., Lea, P. J., Azevedo, R. A. (2002). Activity of antioxidant enzymes in response to cadmium in *Crotalaria juncea*. *Plant and Soil, 239(1),* 123–132. DOI 10.1023/A:1014951524286.
25. Nahar, K., Hasanuzzaman, M., Alam, M. M., Rahman, A., Suzuki, T. et al. (2016). Polyamine and nitric oxide crosstalk: antagonistic effects on cadmium toxicity in mung bean plants through upregulating the metal detoxification, antioxidant defense and methylglyoxal detoxification systems. *Ecotoxicology and Environmental Safety, 126,* 245–255. DOI 10.1016/j.ecoenv.2015.12.026.

26. Rellan-Alvarez, R., Ortega-Villasante, C., Alvarez-Fernandez, A., del Campo, F. F., Hernandez, L. E. (2006). Stress responses of *Zea mays* to cadmium and mercury. *Plant and Soil, 279(1–2),* 41–50. DOI 10.1007/s11104-005-3900-1.
27. Zouari, M., Ben Ahmed, C., Elloumi, N., Bellassoued, K., Delmail, D. et al. (2016). Impact of proline application on cadmium accumulation, mineral nutrition and enzymatic antioxidant defense system of *Olea europaea* L. cv Chemlali exposed to cadmium stress. *Ecotoxicology and Environmental Safety, 128,* 195–205. DOI 10.1016/j.ecoenv.2016.02.024.
28. Howladar, S. M. (2014). A novel *Moringa oleifera* leaf extract can mitigate the stress effects of salinity and cadmium in bean (*Phaseolus vulgaris* L.) plants. *Ecotoxicology and Environmental Safety, 100,* 69–75. DOI 10.1016/j.ecoenv.2013.11.022.
29. Saidi, I., Ayouni, M., Dhieb, A., Chtourou, Y., Chaïbi, W. et al. (2013). Oxidative damages induced by short-term exposure to cadmium in bean plants: protective role of salicylic acid. *South African Journal of Botany, 85,* 32–38. DOI 10.1016/j.sajb.2012.12.002.
30. Carrasco-Gil, S., Estebaranz-Yubero, M., Medel-Cuesta, D., Millán, R., Hernández, L. E. (2012). Influence of nitrate fertilization on Hg uptake and oxidative stress parameters in alfalfa plants cultivated in a Hg-polluted soil. *Environmental and Experimental Botany, 75,* 16–24. DOI 10.1016/j.envexpbot.2011.08.013.
31. Zhang, F., Wan, X. Q., Zhong, Y. (2014). Nitrogen as an important detoxification factor to cadmium stress in poplar plants. *Journal of Plant Interactions, 9(1),* 249–258. DOI 10.1080/17429145.2013.819944.
32. Tischner, R. (2000). Nitrate uptake and reduction in higher and lower plants. *Plant, Cell and Environment, 23(10),* 1005–1024. DOI 10.1046/j.1365-3040.2000.00595.x.
33. Hu, P., Yin, Y. G., Ishikawa, S., Suzui, N., Kawachi, N. et al. (2013). Nitrate facilitates cadmium uptake, transport and accumulation in the hyperaccumulator *Sedum plumbizincicola*. *Environmental Science and Pollution Research, 20(9),* 6306–6316. DOI 10.1007/s11356-013-1680-3.
34. Zaccheo, P., Crippa, L., Pasta, V. D. M. (2006). Ammonium nutrition as a strategy for cadmium mobilisation in the rhizosphere of sunflower. *Plant and Soil, 283(1–2),* 43–56. DOI 10.1007/s11104-005-4791-x.
35. Hernández-Gómez, E., Valdez-Aguilar, L. A., Cartmill, D. L., Cartmill, A. D., Alia-Tajacal, I. et al. (2015). Supplementary calcium ameliorates ammonium toxicity by improving water status in agriculturally important species. *AoB Plants, 7,* 1–8. DOI 10.1093/aobpla/plv105.
36. Helali, M. S., Nebli, H., Kaddour, R., Mahmoudi, H., Lachaâl, M. et al. (2010). Influence of nitrate-ammonium ratio on growth and nutrition of *Arabidopsis thaliana*. *Plant and Soil, 336(1–2),* 65–74. DOI 10.1007/s11104-010-0445-8.
37. Ten, H. F., Cuin, T. A., Pedas, P., Hegelund, J. N., Shabala, S. et al. (2010). Competition between uptake of ammonium and potassium in barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences. *Journal of Experimental Botany, 61(9),* 2303–2315. DOI 10.1093/jxb/erq057.
38. Crawford, N. M., Glass, A. D. M. (1998). Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Science, 3(10),* 389–395. DOI 10.1016/S1360-1385(98)01311-9.
39. Hu, Y., Wang, N. S., Hu, X. J., Lin, X. Y., Feng, Y. et al. (2013). Nitrate nutrition enhances nickel accumulation and toxicity in *Arabidopsis* plants. *Plant and Soil, 371(1–2),* 105–115. DOI 10.1007/s11104-013-1682-4.
40. Liao, M. J., Yu, F. H., Song, M. H., Zhang, S. M., Zhang, J. Z. et al. (2003). Plasticity in R/S ratio, morphology and fitness-related traits in response to reciprocal patchiness of light and nutrients in the stoloniferous herb, *Glechoma longituba* L. *Acta Oecologica, 24(5–6),* 231–239. DOI 10.1016/j.actao.2003.07.001.
41. Monostori, P., Wittmann, G., Karg, E., Turi, S. (2009). Determination of glutathione and glutathione disulfide in biological samples: an in-depth review. *Journal of Chromatography B, 877(28),* 3331–3346. DOI 10.1016/j.jchromb.2009.06.016.
42. Wang, H. P., Schafer, F. Q., Goswami, P. C., Oberley, L. W., Buettner, G. R. (2003). Phospholipid hydroperoxide glutathione peroxidase induces a delay in G_1 of the cell cycle. *Free Radical Research, 37(6),* 621–630. DOI 10.1080/1071576031000088283.
43. Bates, L. S., Waldren, R. P., Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39(1),* 205–207. DOI 10.1007/BF00018060.

44. Beauchamp, C., Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry, 44(1),* 276–287. DOI 10.1016/0003-2697(71)90370-8.
45. Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology, 105,* 121–126.
46. Dabrowski, P., Baczewska, A. H., Pawluskiewicz, B., Paunov, M., Alexantrov, V. et al. (2016). Prompt chlorophyll a fluorescence as a rapid tool for diagnostic changes in PSII structure inhibited by salt stress in *Perennial ryegrass*. *Journal of Photochemistry and Photobiology B: Biology, 157,* 22–31. DOI 10.1016/j.jphotobiol.2016.02.001.
47. Bolhar-Nordenkampf, H. R., Long, S. P., Baker, N. R., Oquist, G., Schreiber, U. et al. (1989). Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology, 3(4),* 497–514. DOI 10.2307/2389624.
48. Butler, W. L., Kitajima, M. (1975). Fluorescence quenching in photosystem II of chloroplasts. *Biochimica et Biophysica Acta, 376(1),* 116–125. DOI 10.1016/0005-2728(75)90210-8.
49. Oquist, G., Chow, W. S. (1992). On the relationship between the quantum yield of photosystem II electron transport, as determined by chlorophyll fluorescence and the quantum yield of CO_2-dependent O_2 evolution. *Photosynthesis Research, 33(1),* 51–62. DOI 10.1007/BF00032982.
50. Maxwell, K., Johnson, G. N. (2000). Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany, 51(345),* 659–668. DOI 10.1093/jexbot/51.345.659.
51. Lichtenthaler, H. K. (1987). Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology, 148,* 350–382.
52. Bankaji, I., Cacador, I., Sleimi, N. (2015). Physiological and biochemical responses of *Suaeda fruticosa* to cadmium and copper stresses: growth, nutrient uptake, antioxidant enzymes, phytochelatin, and glutathione levels. *Environmental Science and Pollution Research International, 22(17),* 13058–13069. DOI 10.1007/s11356-015-4414-x.
53. Jemal, F., Ghorbal, M. H., Zarrouk, M. (2000). Effects of cadmium on lipid composition of pepper. *Biochemical Society Transactions, 28(6),* 907–910. DOI 10.1042/bst0280907.
54. Amani, A. L. (2008). Cadmium induced changes in pigment content, ion uptake, proline content and phosphoenolpyruvate carboxylase activity in *Triticum aestivum* seedlings. *Australian Journal of Basic and Applied Sciences, 2(1),* 57–62.
55. Hammami, S. S., Chaffai, R., Ferjani, E. E. (2004). Effect of cadmium on sunflower growth, leaf pigment and photosynthetic enzymes. *Pakistan Journal of Biological Sciences, 7(8),* 1419–1426. DOI 10.3923/pjbs.2004.1419.1426.
56. Sandalio, L. M., Dalurzo, H. C., Gomez, M., Romero-Puertas, M. C., del Rio, L. A. (2001). Cadmium-induced changes in the growth and oxidative metabolism of peaplants. *Journal of Experimental Botany, 52(364),* 2115–2126. DOI 10.1093/jexbot/52.364.2115.
57. Chugh, L. K., Sawhney, S. K. (1999). Photosynthetic activities of *Pisum sativum* seedlings grown in presence of cadmium. *Plant Physiology and Biochemistry, 37(4),* 297–303. DOI 10.1016/S0981-9428(99)80028-X.
58. McClure, P. R., Kochian, L. V., Spanswick, R. M., Shaff, J. E. (1990). Evidence for cotransport of nitrate and protons in maize roots: I. Effects of nitrate on the membrane-potential. *Plant Physiology, 93(1),* 281–289.
59. Miller, A. J., Cookson, S. J., Smith, S. J., Wells, D. M. (2001). The use of microelectrodes to investigate compartmentation and the transport of metabolized inorganic ions in plants. *Journal of Experimental Botany, 52(356),* 541–549. DOI 10.1093/jexbot/52.356.541.
60. Crawford, N. M., Glass, A. D. M. (1998). Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Science, 3(10),* 389–395. DOI 10.1016/S1360-1385(98)01311-9.
61. Saitoh, T., Seiwa, K., Nishiwaki, A. (2006). Effects of resource heterogeneity on nitrogen translocation within clonal fragments of *Sasa palmata*: an isotopic (^{15}N) assessment. *Annals of Botany, 98(3),* 657–663. DOI 10.1093/aob/mcl147.
62. Li, W., Wang, J. (2011). Influence of light and nitrate assimilation on the growth strategy in clonal weed *Eichhornia crassipes*. *Aquatic Ecology, 45(1),* 1–9. DOI 10.1007/s10452-010-9318-8.