Genetic variation of copper stress tolerance and shoot copper accumulation in Purslane (*Portulaca oleracea*)

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Urban agriculture becomes popular in the United States due to the increasing demands of local foods. However, many urban lands are potentially contaminated by various heavy metals, such as copper, due to human activities and historic use of the lands. These potential contaminations brought up major safety concerns on consumption of the urban produce. Identifying new species or varieties within the species that tolerate heavy metals and accumulate less in their edible part is the key to urban agriculture. Purslane (*Portulaca oleracea*) is considered as the power food of the future due to its high nutritional properties. In this report, we examined the effects of copper stress on seed germination, seedling development and plant growth, as well as genetic diversity of copper accumulation in its edible part among purslane accessions collected from geographically diverse regions. High concentration of $CuSO_4$ does not affect seed germinations for most of accessions except Tokombia and Egypitum. However, inhibition of hypocotyl elongation by copper stress is dose dependent suggesting that it could be a better trait to quantify copper tolerance at germination stage. The copper tolerance of purslane at adult stage is different from that of germination stage. Three purslane accessions, Egypitum, Golden T and Tokombia, showed increased shoot biomass production, while two of these, Egypitum and Golden T, also had an increased root biomass by 600 ppm $CuSO_4$ treatment compared to the control. Genetic variation in copper accumulation in shoots does exist with accessions Egyptium, Eritrea, Tokombia and Turkey accumulating significant less copper in their edible parts. Our results suggest that some purslane accessions have potential to be used in Urban Agriculture to produce safe products for human consumption.

Keywords: purslane; copper stress; seed germination; hypocotyl elongation; plant growth; copper accumulation.

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Introduction

Urban agriculture is very popular around the world, especially in developing countries. It is estimated that more than 200 million urban residents produce food for urban market; it provides about 20% of world's food [1]. In the United States, the practice of urban agriculture is not new; it can be traced back to the economic depression periods of the 1890s [2]. During the World War II, government sponsored urban farms and community gardens were considered patriotic duties [3, 4]. Most of the established gardens have been forgotten due to lack of government support. In recent years, with increasing demands for locally grown produce and driven by a heightened health perspective, the urban agriculture is once again being adopted across the United States. According to the American Community Gardening Association (ACGA) report [5], more than 18,000 urban farms are established in North America producing a variety of crops including fresh fruits and vegetables grown, marketed and consumed by local residents. However, urban soils are often closer to pollution sources, such as industrial areas, waste dumps, and demolition sites, and many soil contaminants are present at higher concentrations in urban areas [6]. One such pollutant is heavy metals, which are known to be harmful to humans when exposed at high dosage. As a result, the consumption of contaminated food can seriously deplete some essential nutrients in the body causing a decrease of immunological defense, disabilities associated with malnutrition and a high prevalence of upper gastrointestinal cancer according to Oliver [7].

Copper is considered as a micronutrient which is essential for plant growth at very low concentrations. Copper serves as co-factor for several proteins and enzymes involved in photosynthesis and respiration [8]. However, excess amount can cause toxicity to plants leading to chlorosis, growth retardation or complete death [9, 10]. Consumption of high dose of copper through contaminated foods causes damages to kidney and liver and may lead to death in human [11]. In the urban areas, soils have high risk to be contaminated by copper [12]. This is because copper pollution in soils links with human activities. For example, many manufacturing companies use copper as one of compounds to produce pesticides, fungicides, and electrical appliances. In addition, heavy use of these pesticides and fungicides in resident areas also releases more copper compounds into the urban environments [13]. Therefore, copper contamination in the urban areas is one of major challenges for urban agriculture and raises safety concerns for consumption of foods produced on urban lands. It is important to identify species or varieties within the species that are tolerant to copper for their growth and accumulate less in their edible parts when growing on such contaminated lands.

Purslane, *Portulaca oleracea*, is one of most resilient species that grows in hostile environmental conditions worldwide [14]. It has been identified as one of the richest vegetable sources of omega-3 fatty acids [15, 16]. Additionally, purslane is also rich in Vitamin A and C, Calcium, Phosphorus, and Iron [14, 17]. It also enriches in antioxidants such as melatonin [18]. Its nutritional characteristics have drawn much attention, and scientists claimed that purslane be the power food of the future [19, 20]. Actually, in recent years, more and more people consumed purslane as a vegetable, and many recipes involving purslane are published online. Aside from its nutritional values, purslane has also been widely used for medicinal plants worldwide [21, 22]. Therefore, it would be interesting to evaluate purslane for its potential as a domesticated vegetable to grow on urban lands. Taking the advantages of our collected purslane accessions worldwide, the objectives of this study are to (1) examine the effect of copper stress on seed germination and seedling development; (2) evaluate the effects of copper stress on biomass production for both shoot (edible part) and root; and (3) investigate the accumulation of copper in in the edible parts when grown on copper treated soils.

Materials and Methods

Plant materials

Nine purslane accessions collected worldwide, including P.O. (*P. oleracea*, a wild accession), P.S. (*P. oleracea* subsp. Sativa, a wild accession) and Turkey (from Turkey), Eritrea and Tokombia (from Eritrea), Golden G and Golden T (from Netherland), wild Greece (from Greece), and Egyptium (from Egypt), were evaluated for their genetic variations on copper tolerance at both germination and adult stages. The experiments were conducted at the Agriculture Research Station and in the Greenhouse at the Randolph Farm of the Virginia State University.

Effects of copper stress on purslane germination

To test the effect of copper stress on seed germination, seeds of all nine purslane accessions were germinated in Petri-Dishes on three layers of Whatman filter paper saturated with 20 ml of 0, 60, 100, 200, 300, 400, 600, and 800 part per million (ppm) of $CuSO_4$. Excess solutions were removed and at least 50 seeds were spread on each petri dish. For statistical analysis, the experiment was triplicated. All petri dishes containing purslane seeds were maintained in a growth chamber at 23°C and light intensity at 120 µmol/m^2 and a 14h/10h light/dark photoperiod. The relative seed germination rates were calculated after an 8-day-germination period.

Inhibitory effect of copper stress on purslane hypocotyl elongation

After germination data collected from the above mentioned experiment, hypocotyl length in millimeters were measured for each treatment. Ten germinated seedlings from each Petri Dish were randomly selected and hypocotyl length was measured and recorded. The average of the 10 measured hypocotyl length from each petri dish was considered as one data and treatments were triplicated for statistical analysis.

Evaluation of purslane adult response to copper stress

To evaluate copper stress response at the adult stage, seeds from all nine purslane accessions were grown in 7'' pots filled with Redi-Earth Plug Mix in greenhouse under natural light condition. For each accession, a total of 6 pots were grown and after germination, 5 seedlings in each pot were saved. After a four-week growth period, pots containing seedlings for each accession were divided into two groups with three pots in each group. One group for all accessions were saturated with water and set as a control and the other group was saturated with 600 ppm $CuSO_4$. The treatment was repeated once every week for two weeks. Data were collected after additional two-week growth period starting from the first day of the treatment.

To evaluate effect of the copper on purslane development, individual plants from each treatment per accession were harvested. Both shoots and roots were thoroughly rinsed and allowed air dry for two hours on paper towels. Fresh shoots and roots biomass were then evaluated in a triplicate design.

Evaluation of copper accumulation in purslane above-ground tissues

To examine the accumulation of copper in the shoots after $CuSO_4$ treatment, the harvested shoots were thoroughly rinsed to avoid surface contamination. The cleaned tissues were then dried at a 70°C oven for three days. Shoots collected from the same pot were pooled together and served as one replicate. The dried tissues were then grinded with a miller and subjected to analyze copper contents following the method recommended by USEPA protocol of SW-846 (https://www.epa.gov/hw-sw846). The measurement was conducted at the Waypoint Analytical Virginia and analysis was conducted as triplicate design.

Statistical analysis

Student T-test and Duncan multiple test were used for statistical analysis where is applicable. $P<0.05$ was used as cut off for significant differences among between treatment and among accessions.

Results

Effect of copper stress on seed germination in purslane

Seeds of all nine purslane accessions were germinated on petri dishes with control (water only) and a series of concentrations of $CuSO_4$ (60, 100, 200, 300, 400, 600, and 800 ppm). Relative germinated rates were calculated and shown in Figure 1. Copper stress, even at 800 ppm level, did not affect seed germinations for 7 out of 9 accessions, including Eritrea, Golden G, Golden T, Greece, P.O., P.S., and Turkey (Figure 1B). Only two accessions Egyptium and Tokombia were significantly affected by copper stress (Figure 1A, 1B). At lower Cu concentration (below 300 ppm), even for accessions of Egyptium and Tokombia, their germination rates were not affected by copper stress. Only when Cu concentration raised to above 400 ppm, their germination rates were abruptly dropped and significantly inhibited (Figure 1A, 1B).

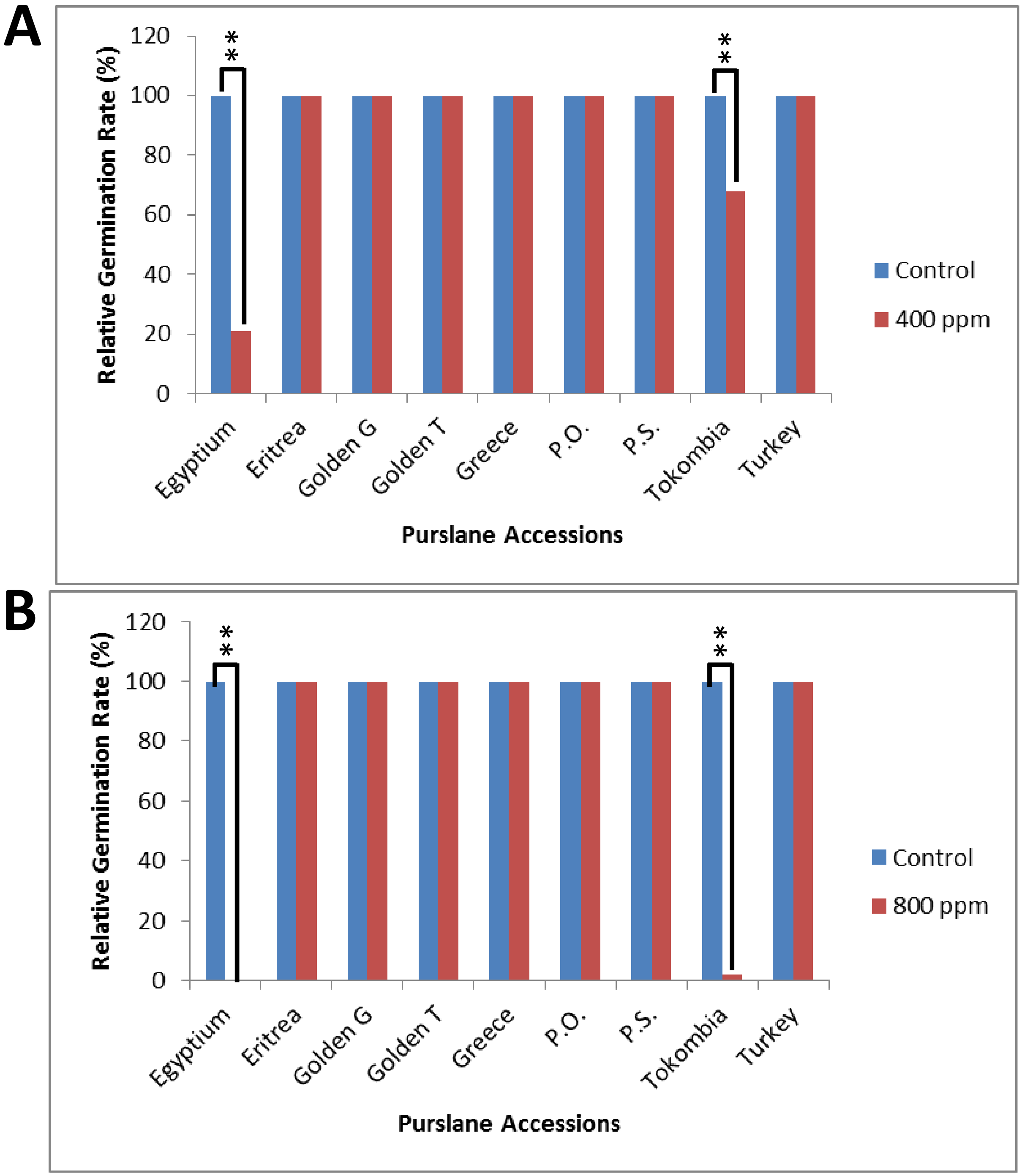

Figure 1. Effect of copper stress on purslane seed germination. Nine purslane accessions were evaluated for their germination responses to different copper stresses. **A.** Relative germination rates of purslane accessions in response to 400 ppm $CuSO_4$; **B.** Relative germination rates of purslane accessions in response to 800 ppm $CuSO_4$. ** represents a significant difference at P<0.01 level in T-test.

Copper stress significantly inhibits hypocotyl elongation

Even though seed germination was not affected by copper stress for most of purslane accessions (Figure 1), hypocotyl elongation after germination was significantly inhibited by copper stress. Treatment with 60 ppm Cu did not inhibit hypocotyl elongation for most of accessions and for some accessions, this concentration of Cu even enhanced elongation (data not shown). However, at the 100 ppm copper level, hypocotyl elongation was significantly inhibited for most of accessions (Figure 2A). When copper concentration increased to 600 ppm, the significant inhibition effect can be observed for all purslane accessions (Figure 2B). The most reduction in absolute hypocotyl length is for accession Golden T and P.S. (Figure 2B), while the least reduction is for accession Golden G. In general, the reduction rate in hypocotyl length is dose dependent for 7 out 9 accessions. However, for accessions Tokombia and Egyptium, such inhibition dropped dramatically at lower Cu concentration (100 ppm) and continuously kept low at higher Cu concentrations. Figure 2C showed such trends for 4 of the represented accessions. These results strongly suggest that hypocotyl length is a better trait than germination rate in evaluating copper stress tolerance at seedling stage in purslane. Accordingly, we identified that Tokombia and Egyptium are most sensitive to copper stress among 9 purslane accessions at seedling stage, and accession Golden G is most tolerance to copper stress at seedling stage.

Response of purslane accessions to copper stress at adult stage

Due to the high sensitivity of accessions Tokombia and Egypitum to copper stress in seed germination and hypocotyl elongation stage, to avoid a complete death of these two accessions, we evaluated the effect of Cu stress on purslane plant development by treating purslane plants at 4-week growing stage with 600 ppm $CuSO_4$ once a week for continuous two weeks. We first evaluated the effect of copper stress on fresh shoot biomass production. As shown in Figure 3A, 600 ppm $CuSO_4$ treatment only significantly reduced shoot biomass for accession Greece. The treatment also reduced biomass for accessions Eritrea and Golden G, but statistically not significant. To our surprise, the treatment significantly increased shoot biomass production for three purslane accessions: Egypitum, Tokombia, and Golden T (Figure 3A). The shoot biomass production almost doubled for accessions Egypitum and Golden T comparing to their untreated controls (Figure 3A).

Further examination was conducted on the effect of $CuSO_4$ treatment on root fresh biomass production. No significant differences were observed for most of accessions. However, 600 ppm $CuSO_4$ treatment significantly increased root biomass production for accessions Egypitum and Golden T, and the amount of root biomass for Egypitum more than doubled comparing to that of control treatment (Figure 3B). The trend of increasing root biomass for Egypitum and golden T are consistent with that of shoot biomass production. On the contrary, not like that of shoot biomass production, the root biomass production for accession Tokombia was significantly reduced. These results indicate that 600 ppm $CuSO_4$ seemed not a stress to accessions Egypitum and Golden T, instead, such treatment significantly enhanced biomass production for both shoots and underground tissues. However, for the accession Tokombia, 600 ppm $CuSO_4$ treatment altered its energy re-distribution between shoot and root. Under such stress condition, it took more energy to enhance shoot biomass production, and less energy was contributed to produce root biomass (Figure 3A and 3B). Consistent with these observations, when comparing shoot/root ratio between treatment and the corresponding control, the significant difference was only observed for the accession Tokombia and no statistical difference was identified for all other purslane accessions (Figure 3C).

Shoot copper accumulation in different purslane accessions

Although purslane is a wild species, its high nutritive and antioxidant properties make it being considered as the 'power food for the future' [19, 20]. Actually, in recent years, many people consumed purslane as vegetable, and more and more food recipes involving purslane are developed. Therefore, for the safety concerns, it would be interest to examine copper

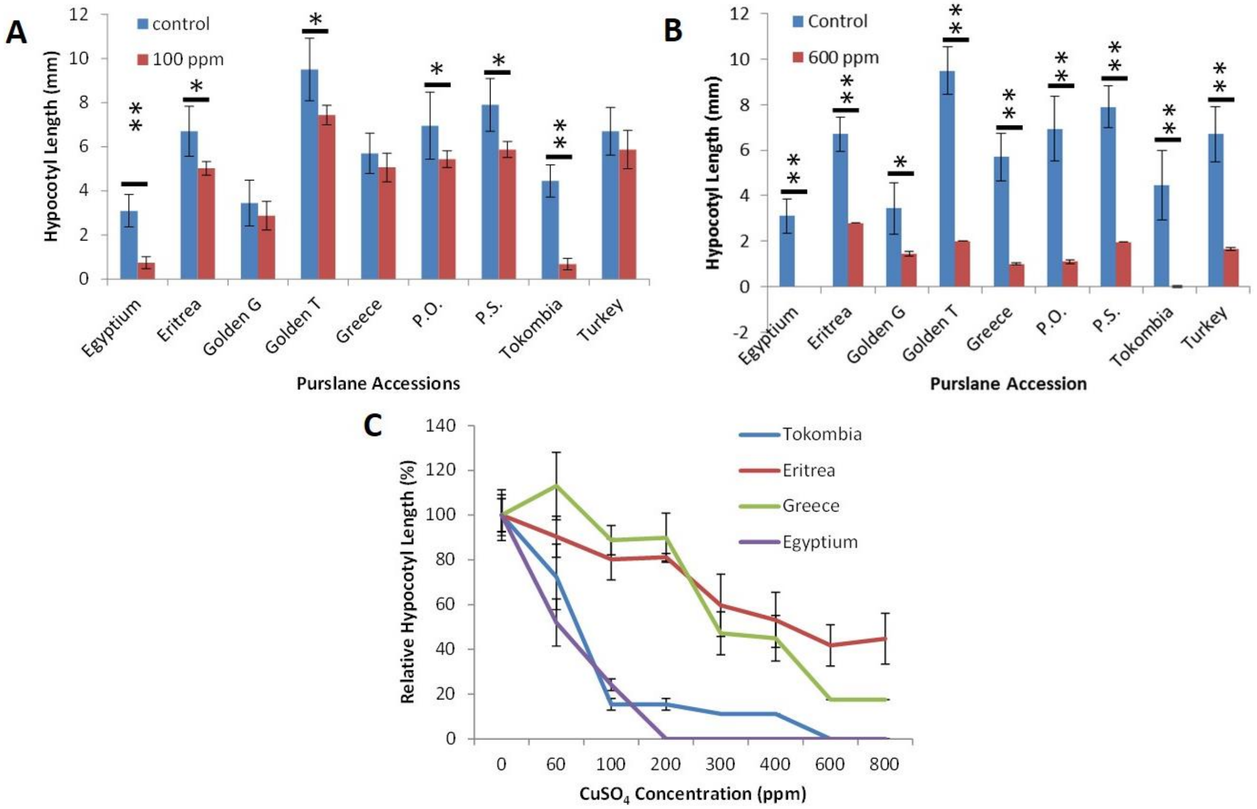

Figure 2. Hypocotyl elongation affected by copper stress. **A.** Inhibition of hypocotyl elongation by 100 ppm $CuSO_4$; **B.** Inhibition of hypocotyl elongation by 600 ppm $CuSO_4$; **C.** Responses of four purslane accessions to different copper stresses (from 60 ppm to 800 ppm). * represents a significant difference at $P<0.05$ level and ** represents a significant difference at $P<0.01$ level in T-tests.

accumulation in their edible part and identify accessions with low accumulation of copper. We examined shoot copper accumulation for all nine purslane accessions under high copper stress condition, and the result is shown in Figure 4. Overall, under copper treated condition, purslane accessions accumulated Cu in their shoots ranging from 11 mg/kg to 37 mg/kg dry tissue. Accessions Egypitum, Eritrea, Turkey, and Tokombia accumulated least Cu in their shoots (all below 14 mg/kg dry tissue), while accessions Greece and PS accumulated the most with more than 34 mg/kg dry tissue. These results suggest that genetic variation on shoot copper accumulation do exist among purslane accessions.

Discussion

Purslane (*P. oleracea*) is considered as a wild species and is highly adaptable to many hostile environmental conditions such as heat, drought, salt and nutrient-deficient conditions [14]. Due to its high nutritive value and antioxidant properties, purslane was defined as the 'power food for the future' [19, 20]. In many regions of the world, such as Europe, the Middle East, Asia, and South America, purslane is consumed as a leafy vegetable [17, 23, 24]. For example, purslane is an important component in a Mediterranean diet, especially in Greece and Turkey [23, 24]. In recent years, more and more Americans adapted purslane in their diet and many recipes are developed and posted online. Addition to its consumption as food, purslane has also been considered as medicinal plant in many countries. The most well-known medicinal properties of purslane are its antimicrobial [25],

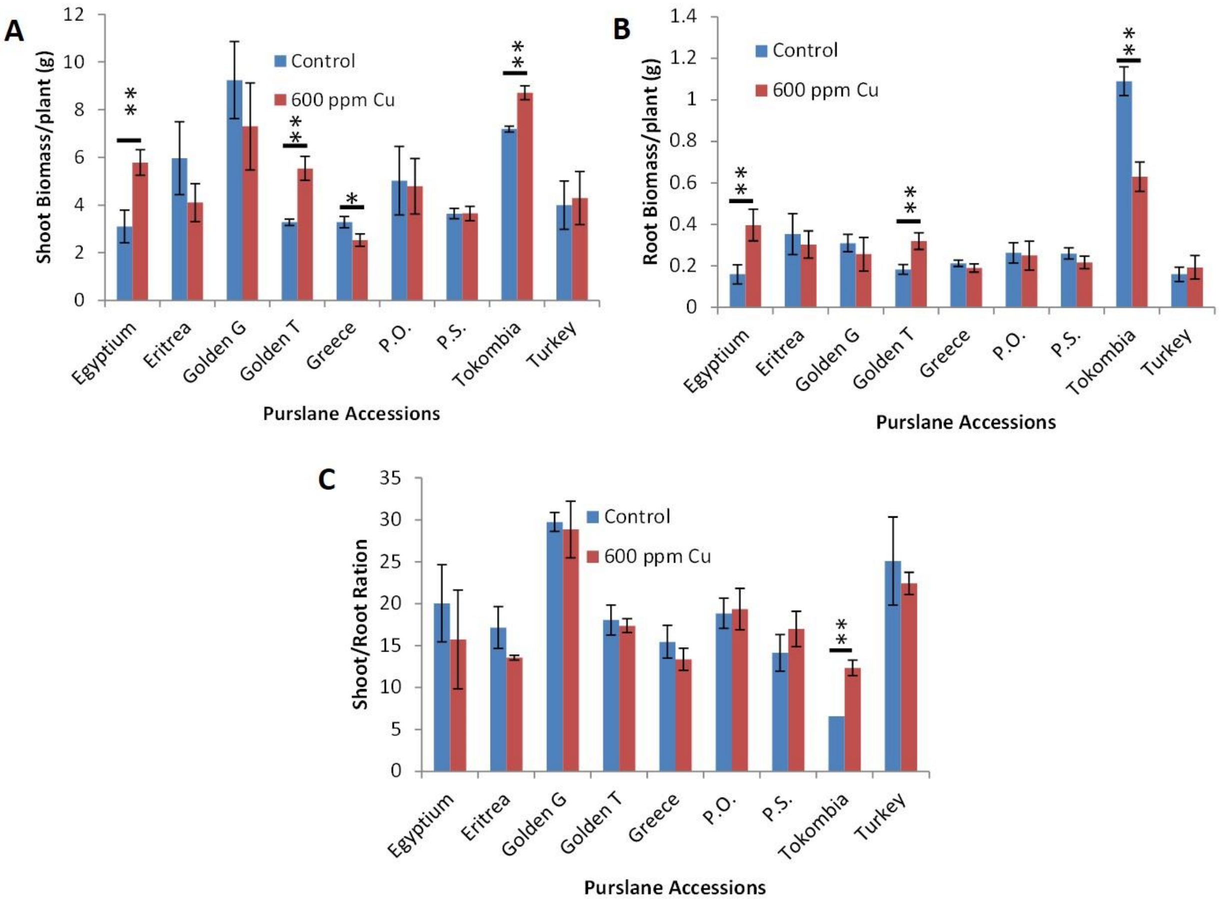

Figure 3. Biomass production affected by 600 ppm copper stress. **A.** Shoot biomass production of nine purslane accessions after 2-wk 600 ppm $CuSO_4$ treatment; **B.** Root fresh biomass accumulation of 9 purslane accessions after 2-wk 600 ppm $CuSO_4$ treatment; **C.** Shoot/root ratio of purslane accession to depict energy redistribution due to copper stress. * represents a significant difference between control and treatment at $P<0.05$, and ** represents a significant difference between control and treatment at $P<0.01$.

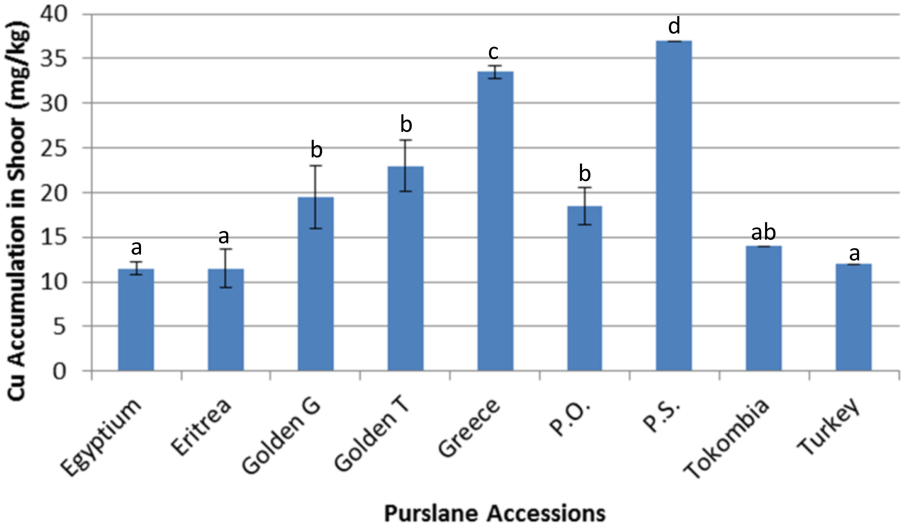

Figure 4. Genetic variation of copper accumulation in shoots of purslane treated with 2-wk period of 600 ppm $CuSO_4$. Different letters indicate a significant difference (Duncan Test at $P<0.05$) between accessions.

anti-inflammatory [26, 27], wound healing [28], and anti-ulcerogenic effect [29].

In this report, we examined the genetic variations in copper tolerance among P. oleracea accessions collected from geographically different regions worldwide. When $CuSO_4$ concentration was lower than 300 ppm, all purslane accessions germinated with no difference to the control. Even $CuSO_4$ concentration was raised to 800 ppm (which is equivalent to about 5 mM of $CuSO_4$), 7 out nine accessions still had similar germination rate comparing to the control treatment. Only two accessions, Tokombia and Egyptium, responded to copper stress in terms of germination rate. When $CuSO_4$ concentration reached to 400 ppm, it significantly inhibited germination for both accessions. When $CuSO_4$ concentration increased to 800 ppm, it almost completely blocked the germination for Tokombia and Egyptium.

We also examined variations in copper tolerance by evaluating the effect of copper stress on hypocotyl elongation during germination stage. Different from seed germination responding to copper stress, hypocotyl elongation in purslane is much sensitive to copper stress. Even at 100 ppm Cu level, 6 out of 9 accessions showed significant inhibition for their hypocotyl elongation (Figure 2A). In addition to accessions Tokombia and Egypitum, which also showed significant sensitivity in seed germination, four other accessions (Eritrea, Golden T, P.O., and P.S.) also demonstrated high sensitivity to copper stress. The inhibition effect on hypocotyl elongation strengthened when $CuSO_4$ concentration increased, and at 600 ppm level, significant inhibition was observed for all 9 accessions. Furthermore, the inhibition effect showed dosage dependent for 7 out of 9 accessions. Only accessions Tokombia and Egypitum showed significant inhibition at very low level of copper stress and kept low with increase of $CuSO_4$ concentration (Figure 2C). Taken together, our results suggest that Tokombia and Egyptium be the most sensitive to copper stress during seed germination stage. The data also indicate that hypocotyl elongation may be the best trait to evaluate stress tolerance at germination stage. Interestingly, our previous study also identified Tokombia and Egypitum being most sensitive to drought stress during germination stage [30]. Given that both drought stress and copper stress are considered as abiotic stress, our results may suggest that common mechanisms or regulatory pathways control response to general abiotic stress at germination stage in purslane accessions Tokombia and Egypitum.

In general, abiotic stress tolerance at seedling stage does not necessarily translate into adult stage stress tolerance. This is exactly what we observed in our previous study in purslane's drought tolerance [30]. To further understand copper tolerant capacity at adult stage in *P. oleracea*, we measured both shoot and root biomass production and compared the parameters between control and 600 ppm $CuSO_4$ treatment. Significant genetic variation was observed in shoot biomass production in responding to $CuSO_4$ treatment (Figure 3A). For three accessions, Tokombia, Egypitum, and Golden T, we found that 600 ppm $CuSO_4$ treatment did not inhibit plants' growth, instead, the treatment significantly increased biomass production comparing to the control. Copper is considered as a micronutrient and is essential for plant growth and development [31], however, excess amount of copper would cause stress and inhibit plants' growth and development [32]. The fact that 600 ppm $CuSO_4$, which is equivalent to 3.8 mM $CuSO_4$, did not cause any stress at adult stage to above mentioned three purslane accessions suggest that it must exist an unique mechanism in these accessions that allow plants efficiently use Cu as micronutrient and prevent excess amount to inhibit plant's development. Interestingly, when root parameter was measured, we found that two of these three accessions, Egypitum and Golden T, also had significant more root biomass produced in $CuSO_4$ treated group comparing to the control (Figure 3B). On the contrary, for accession Tokombia, it significantly reduced root biomass production comparing the control group. Preciously we found that accessions Tokombia and Egypitum used different mechanisms to fight against drought stress [30]. Our new study also demonstrated that different mechanisms exist between Tokombia and Egypitum in responding to copper stress.

It is also interesting to note that accession Tokombia is unique in root development comparing to other accessions. This observation is true under both control and $CuSO_4$ treatment conditions (Figure 3B). This result is consistent with our previous discovery [30]. Such unique root system may be the key for plants defending against abiotic stresses.

Purslane is one of the richest vegetable sources of omege-3 fatty acids [15, 16]. It also contains high amount of Vitamin A, Vitamin C, Calcium, Phosphorus, iron [14, 17], alpha tocopherol [17], and melatonin [18]. Because of these, it is considered as the 'power food of future' [19, 20]. In addition, due to the nature of this species, purslane can be adapted to different environmental conditions [33-36]. Therefore, it provides a unique opportunity to produce high quality food for human consumption and reduce agricultural use of precious water resources in agriculturally marginal lands. In recent years, urban agriculture becomes popular in the United States due to the increasing demands of local foods. However, many urban areas are potentially contaminated by various heavy metals, such as copper due to the historic use of the lands. Identifying crops (species) that can grow in urban areas and safe enough to be consumed by local residents is an important research topic. In order to search new crops that can be grown in urban lands, we quantified Cu accumulation in the edible part (shoots) of purslane under $CuSO_4$ treatment condition. Our results showed that Cu accumulation in shoots in purslane are highly variable, ranging from 11 mg/kg dry mass to 37 mg/kg dry mass. At least four accessions accumulated less than 15 mg/kg Cu in their edible part. Even though, we did not quantify Cu accumulation in our control group, such accumulation range under copper treatment is similar to that of purslane without additional copper treatment according to report in literature [37]. Therefore, these four accessions could be used to grow on urban areas with Cu contamination in soil and produce quality food for human consumption.

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