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Comparative Arsenic Tolerance and Accumulation Potential between Wild *Tagetes patula* and *Tagetes minuta*

(Perbandingan Potensi Toleransi dan Pengumpulan Arsenik antara *Tagetes patula* dan *Tagetes minuta Liar*)

Irum Shahzadi[1,*], Mohammad Maroof Shah[1], Irrum Saleem Akhtar[1], Tariq Ismail[2], Raza Ahmad[1], Ismat Nawaz[3], Maria Siddique[4], Sofia Baig[5], Ayesha Baig[1] & Ummara Waheed[6]

[1]*Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, 22060, Pakistan*
[2]*Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, 22060, Pakistan*
[3]*Department of Biosciences, COMSATS University Islamabad, 45550, Pakistan*
[4]*Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus, 22060, Pakistan*
[5]*Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology, H-12, Islamabad, 44000, Pakistan*
[6]*Institute of Plant Breeding & Biotechnology, MNS University of Agriculture, Multan, Pakistan*

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ABSTRACT

Arsenic (As) is a bioactive metalloid that is highly toxic to humans, animals, and plants. Environmental contamination of As especially in groundwater increases due to natural and anthropogenic activities. The present study was performed to evaluate the potential of wild *Tagetes* species for the phytoremediation of As contaminated soil/water. This comparative research aims to analyze As accumulation and tolerance in two wild species of *Tagetes*, *T. minuta* and *T. patula.* The 20 days old seedlings were grown hydroponically and exposed to the different concentrations of As, 0, 50, 150, and 300 µM As_2O_3 for 1-, 4- and 7- days intervals. Effect of As stress was measured on the rate of seed germination, growth parameters like fresh and dry biomass weight, root/shoot length, chlorophyll contents and As contents in root and shoot in both *Tagetes* species. Increasing concentration of As restricts the growth activity of *T. minuta* with toxicity symptoms on leaves such as chlorosis. Accumulation of As in the shoot was significantly ($p \leq 0.01$) high (634 µg g^{-1} DW) in *T. patula* as compared to *T. minuta* (397 µg g^{-1} DW) at 300 µM As_2O_3. Both *Tagetes* species exhibited high variation for As tolerance parameters as well as for As accumulation patterns. Comparatively good tolerance and accumulation of As in *T. patula* suggests that this species could be used in phytoextraction and re-vegetation in As contaminated sites.

Keywords: Arsenic; phytoremediation; *Tagetes minuta; Tagetes patula*

ABSTRAK

Arsenik (As) ialah metaloid bioaktif yang sangat toksik kepada manusia, haiwan dan tumbuhan. Pencemaran persekitaran disebabkan As terutamanya pada air dalam tanah meningkat disebabkan oleh aktiviti semula jadi dan antropogen. Kajian ini dilakukan untuk menilai potensi spesies *Tagetes* liar sebagai fitoremediasi tanah/air yang tercemar As. Kajian perbandingan ini bertujuan untuk menganalisis pengumpulan dan kerintangan As dalam dua spesies *Tagetes* liar, *T. minuta* dan *T. patula*. Anak pokok berusia 20 hari telah ditanam secara hidroponik dan didedahkan kepada kepekatan As berbeza iaitu 0, 50, 150 dan 300 µM As_2O_3 untuk selang 1-, 4- dan 7 hari. Kesan tekanan As diukur dengan melihat kepada kadar percambahan biji benih, parameter pertumbuhan seperti berat biojisim segar dan kering, panjang akar/pucuk, kandungan klorofil dan kandungan As dalam akar dan pucuk pada kedua-dua spesies *Tagetes*. Peningkatan kepekatan As menyekat aktiviti pertumbuhan *T. minuta* dengan gejala ketoksikan seperti klorosis pada daun. Pengumpulan As dalam pucuk *T. patula* adalah tinggi (634 µg g^{-1} DW) dan berbeza secara bererti ($p \leq 0.01$) berbanding *T. minuta* (397 µg g^{-1} DW) pada 300 µM As_2O_3. Terdapat variasi yang tinggi bagi parameter berkait kerintangan As begitu juga dengan corak pengumpulan As pada kedua-dua spesies *Tagetes*. Kerintangan dan pengumpulan As dalam *T. patula* yang agak baik menunjukkan bahawa spesies ini boleh digunakan dalam fitoekstraksi dan boleh ditanam di lokasi tercemar As.

Kata kunci: Arsenik; fitoremediasi; *Tagetes minuta; Tagetes patula*

Introduction

Heavy metals in the environment are due to natural sources such as soil erosion, weathering of minerals, and volcanic eruption and going to rise due to synthetic sources such as mining, fertilizers, pesticides, smelting, sewage, and disposal of industrial waste (Chung et al. 2014; Mandal 2017). In recent years, As received much attention because it contaminates soil and water resulting in environmental pollution that is highly toxic to humans, animals and plants. Although it is a nonessential metalloid and toxic to plants, animals and humans even at a very low level (Chung et al. 2014; Nahar et al. 2017). In the environment, As is mostly present in two forms, organic and inorganic. The Arsenate [As(V)], arsenite [As(III)] and methylated arsenic (MMA, DMA) are the inorganic As species mainly present in soil/water bodies (Li et al. 2016). All of them are toxic and highly carcinogenic. As(V), the dominant form in the soil is taken up by plants via phosphate transporters across the plasma membrane due to the analogous nature of its chemical structure with that of phosphate where it disturbs the phosphate transport system and inhibit the enzymes and protein activity (Leblanc et al. 2013; Tripathi et al. 2007). Similarly, As interrupts the various biochemical and physiological processes of plants (Abbas et al. 2018; Abid et al. 2021; Stoeva et al. 2003) resulting in a decrease in biomass production and root shoot length (Abid et al. 2021; Li et al. 2007), decrease in chlorophyll and carotenoids content due to reactive oxygen species (ROS) formation (Angulo-Bejarano et al. 2021; Chandrakar et al. 2016).

Several techniques including chemical, physicochemical and thermal are being used to clean up the environment from As contaminants but they are costly, technically difficult and change the physiochemical nature of soil and water (Hashim et al. 2011; Singh et al. 2015). Biological techniques especially phytoremediation is an environmentally sound, cost-effective and promising method for the removal of As from soil and water (Angulo-Bejarano et al. 2021; Elisa et al. 2020; Wiszniewska 2021). The efficiency of phytoremediation is based on the capacity of selected plants to grow, tolerate, accumulate and immobilize or degrade the pollutants (Abid et al. 2021; Li et al. 2020; Nahar et al. 2017). The use of wildly growing indigenous plant species such as *T. patula* and *T. minuta* which are naturally growing in metal-contaminated environments is highly desired for the phytoremediation of such contaminated water and soil sites (Sathya et al. 2020).

Tagetes species (from the Asteraceae family) are popularly known as marigold and locally named gul-e-sad burga (Shahzadi et al. 2010). It is originated from South America with a few naturalized populations around the world. Plant species of genus *Tagetes* are growing in a broad spectrum of climate starting from tropical to extreme temperate regions of the world. *Tagetes* species are annual herbs with the plant height ranging from 0.5 to 3.0 m bearing flowers used for ornamental purposes. The wild species of marigold have a well-developed root system and their propagation rate is high due to their attractive flower fragrance toward pollinators (Sathya et al. 2020; Wei et al. 2012). Two wild *Tagetes* species (*T. minuta* and *T. patula*) were selected for the assessment of their potential to phytoextract As artificially provided to them in this study. These wild species are grown widely on the disturbed land along sides the road of Abbottabad, Pakistan and there is no utilization of the produced biomass which is mostly gone to waste. These wild marigold species grow well in poor soils (Coelho et al. 2017) and similarly, during the initial growth phase, marigolds show rapid phytoextraction of heavy metals from the soil and sediments (Choudhury et al. 2016). Marigolds are also used for phytoremediation of crude oil contaminated soil (Salim et al. 2020). Marigolds are a good choice due to these properties for the decontamination of soil (Choudhury et al. 2016). Similarly, for phytoremediation of heavy metal from the contaminated soils and water bodies, different species of marigold plants have been employed.

The phytoextraction and phytoremediation ability of plants is best evaluated by the hydroponic method and this system is appropriate to access the plant metal tolerance and accumulation. Few studies of cultivated ornamental hybrid species of *Tagetes* (*T. eracta* and *T. patula*) are available on tolerance and accumulation of As under pot soil system (Chintakovid et al. 2007) but to the best of our knowledge, no study dealing with physiological, morphological, and biochemical analysis under hydroponics are reported in the literature for the two wild marigolds (*T. minuta* and *T. patula*). In this work, we conducted a hydroponic experiment to compare the physiology of wild *T. minuta* and *T. patula* for their As tolerance and accumulation, to evaluate their potential for the subsequent utilization of phytoremediation purposes.

Materials and Methods

Plant materials, treatment conditions and seed germination

T. minuta and *T. patula* seeds were collected from hilly areas of Abbottabad, KPK province of Pakistan. Seeds were surface sterilized in 75% ethanol for 2 min. They

were then washed with distilled water before germination on a moist cotton bed and watered with 10% Hoagland nutrient medium and put in dark for 2 days. Seedlings were moved to a growth chamber with day, night temperatures 25 ± 2 ℃ and 70% relative humidity for 16 h photoperiod and 8 h dark period. After germination, 20 days old plants were treated with different concentrations of As (0, 50, 150, and 300 µM, using salt As_2O_3) for 1-, 4- and 7- days intervals. Each experiment was conducted in triplicates and each replicate contained an equal size and an equal number of seedlings. After As exposure, the treated plants were harvested for analysis of physiological parameters.

The seed germination rate was also analyzed at all concentrations of As (0, 50, 150, and 300 µM). The experiment was conducted in autoclaved glass petri plates on a cotton bed. The 10 mL each As concentration solution was added to each petri plate and control contained 10 mL of sterile autoclaved water. The three replicate plates were prepared for these different As concentrations and each petri plate contained ten seeds with equal distance. These plates were covered with aluminum foil and kept at room temperature (25 ± 2 ℃) for 14 days. The number of seed germination was counted every two days.

MORPHOLOGICAL ANALYSIS AND GROWTH PARAMETERS

Toxicity symptoms like chlorosis/necrotic spots were measured, for all samples. After harvesting, each plant was separated into leaf, stem and root. Roots were dipped in 0.01 M solution of HCl for 5 min to eliminate the adsorbed metal on the surface of roots and then rinsed thrice with distilled water. Leaves and stems were also thoroughly washed with distilled water. Growth parameters such as seed germination, length of root and shoot (stem and leaf), fresh weight of root and shoot were calculated for all samples.

TISSUE WATER CONTENTS AND TOLERANCE INDEX (T.I)

The tissue water content (TWC) was estimated on the basis of dry weight by the following formula;

$$TWC\ (mL\ g^{-1}\ DW) = (FW - DW)/DW$$

The tolerance index was calculated with the help of the following formula; where T.I is the shoot dry weight of stress plant/shoot dry weight of control plant.

QUANTIFICATION OF TOTAL CHLOROPHYLL

The 0.1 g of leaves were used to estimate chlorophyll a and b contents. The leaves were ground into a fine powder and extracted with acetone (100%). The extracts were then centrifuged for 5 min and analyzed for chlorophyll a and b contents by a UV–Visible spectrophotometer (T80+ UV/VIS spectrometer) at 663 and 645 nm, respectively (Li et 2019; Wellburn 1994). Total chlorophyll was extracted and calculated by Arnon's method (1949).

SAMPLE PREPARATION AND ARSENIC ANALYSIS

Root and shoot (leaf and stem) samples were oven dried at 70 ℃ for 48 h. The dry weights of all samples were recorded. The dried samples (~ 0.1 g) were crushed to a fine powder using pestle-mortar and wet-digested with HNO_3: $HClO_4$ (3:1 v/v). The As concentrations were measured using Atomic Absorption Spectrometer (PerkinElmer Inc., Analyst 700, USA).

PHYTOEXTRACTION CAPACITY

The phytoextraction ability of both *Tagetes* species was assessed using the bioaccumulation factor (BF) and translocation factor (TF), which were enumerated using the following formulas;

where BFis the [As] in shoot / [As] in solution; TF is the[As] in shoot / [As] in root

STATISTICAL ANALYSIS

The data was analyzed using R Program (R Core Team 2018). The triplicate data presented as mean± standard deviations (SD) for each parameter and all experiments were conducted in duplicate. The analysis of variance (ANOVA) was applied followed by Duncan's new multiple range test to determine the significant differences of the As content in control and treated plants. For the principal components analysis (PCA) the principal component extraction was carried out till the Eigenvalues reached 1. The PCs having value component values lower than 1 were considered insignificant. The variables showing higher loading values in component 1, and in component 2 were considered significant. The information related to PC extraction and cumulative variance and component score coefficient matrix is presented in the supplementary material.

RESULTS

MORPHOLOGICAL ANALYSIS AND GROWTH RESPONSE TO ARSENIC STRESS

Increasing As exposure displayed morpho-phytotoxicity symptoms in *T. minuta* as leaf chlorosis but its roots did

not exhibit toxic symptoms. In contrast, such toxicity symptoms were not observed on leaves of *T. patula* even at high As concentrations. The As effect was determined on seed germination and was found to decrease in percent seed germination with increasing As concentration in both species. At the lowest concentration (50 µM), the seed germination rate was observed 90% in *T. minuta* and 60% in *T. patula* (Table 1). At a moderately high concentration of As (150 µM), seed germination rate dropped to 50% in *T. minuta*, whereas a complete suppression of seed germination was observed in both species at the highest As concentration (300 µM) (Table 1).

The ANOVA and Duncan's new multiple range tests on the *T. patula* and *T. minuta* were significant ($p \leq 0.001$) in terms of shoot length, shoot dry weight, root length, root dry weight, tissue water content, tolerance index, chlorophyll content, at different As concentration in shoot and root (Table 2). Non-significant differences were observed in shoot length of *T. patula* and *T.*

TABLE 1. Effect of various As_2O_3 treatments on seed germination rate

Treatment No.	As conc. (µM)	No of seeds (planted per plate)	Seed germination rate (%)	
			T. patula	*T. minuta*
1	0	10	100 ± 0.00^{a}	100 ± 0.00^{a}
2	50	10	60 ± 10.00^{b}	90 ± 10.00^{a}
3	150	10	0	50 ± 10.00^{b}
4	300	10	0	0

TABLE 2. ANOVA results of shoot length, shoot dry weight, root length, root dry weight, tissue water content, tolerance index, chlorophyll content, arsenite concentration in shoot and root of *T. patula* and *T. minuta* in 1-, 4-, 7- day intervals under various concentrations of As treatment

Species	Source of variation	Days	Treatments	Days x Treatments	Residuals	Total
	df	2	3	6	24	35
T. patula	Shoot length	0.407^{ns}	11.614^{***}	5.213^{***}	1.073	--
T. minuta		0.029^{ns}	4.728^{***}	2.617^{***}	0.985	--
T. patula	Shoot dry weight	0.014^{**}	0.345^{***}	0.08^{***}	0.024	--
T. minuta		0.009^{**}	0.214^{***}	0.029^{***}	0.018	--
T. patula	Root length	20.95^{***}	198.85^{***}	24.63^{***}	3.47	--
T. minuta		9.15^{***}	66.14^{***}	16.50^{***}	1.37	--
T. patula	Root dry weight	0.42^{***}	3.58^{***}	0.63^{***}	0.19	--
T. minuta		0.004^{**}	0.099^{***}	0.004^{ns}	0.006	--
T. patula	Tissue water content	243.5^{***}	754.7^{***}	120.1^{***}	80.1	--
T. minuta		30.24^{***}	204.31^{***}	38.86^{**}	36.87	--
T. patula	Tolerance index	0.280^{***}	4.690^{***}	0.156^{***}	0.065	--
T. minuta		0.192^{***}	4.033^{***}	0.096^{**}	0.086	--
T. patula	Chlorophyll content	0.009^{***}	0.208^{***}	0.008^{***}	0.0014	--
T. minuta		0.061^{***}	0.297^{***}	0.028^{***}	0.0002	--
T. patula	Arsenite conc. in shoot	356640^{***}	1046401^{***}	143846^{***}	1657	--
T. minuta		115235^{***}	396563^{***}	52013^{***}	411	--
T. patula	Arsenite conc. in root	90717^{***}	916139^{***}	31524^{***}	1223	--
T. minuta		35299^{***}	491055^{***}	14763^{***}	552	--

*** and ** stands for significant at 0.001 and 0.01 probability levels, ^{ns} for non-significant

minuta between the days and similarly, non-significant differences were also observed in the root dry weight of *T. minuta* between the days and treatment (Table 2). The ANOVA analysis also showed significant differences ($p \leq 0.01$) of shoot dry weight, root dry weight, tissue water content and tolerance index of *T. minuta* between the days and treatment (Table 2).

Shoot length and dry biomass decreased with increasing As concentration in both species (Figure 1(a), 1(c)) with the period of 1-, 4- and 7- days intervals. Shoot length decreased significantly up to 58% (*T. patula*) and 54% (*T. minuta*) at 300 µM As concentration as compared to control plants (Figure1(a)), whereas, reduction in shoot dry biomass at this As stress level, was observed 69 and 66% in *T. patula* and *T. minuta,* respectively (Figure 1(c)) after 7 days. The least decrease in shoot length was 6% in *T. minuta* on day 1 at 300 µM As concentration although it became more obvious over a prolonged period. However, shoot biomass reduction was highest in *T. minuta* in comparison to *T. patula* at 300 µM As concentration on day 1. After the application of As, changes were observed in root length and root biomass of *T. patula* and *T. minuta*. A significant ($p \leq 0.01$) reduction in root biomass and root length was also observed for both species (Figure 1(b), 1(d)). Root length was reduced drastically upon the exposure to As stress (50 µM), however, on increasing As concentration both species maintained their root length, particularly *T. patula* (Figure 1(b)). The reduction in root length was 53-75% in *T. patula* and 20-71% in *T. minuta* from day 1 to day 7 at maximum exposure of As concentration. However, both species sustained their growth at the elevated level of As concentration and according to Duncan's multiple comparison test, *T. patula* showed a significant increase of shoot-biomass in comparison to *T. minuta*. Overall, both species showed a decrease in their root dry biomass, but the reduction of root dry biomass was much higher in *T. patula* (38-65%) and only 20-31% in *T. minuta*, as compared to control from day 1 to day 7 at 300 µM As concentration (Figure 1(d)).

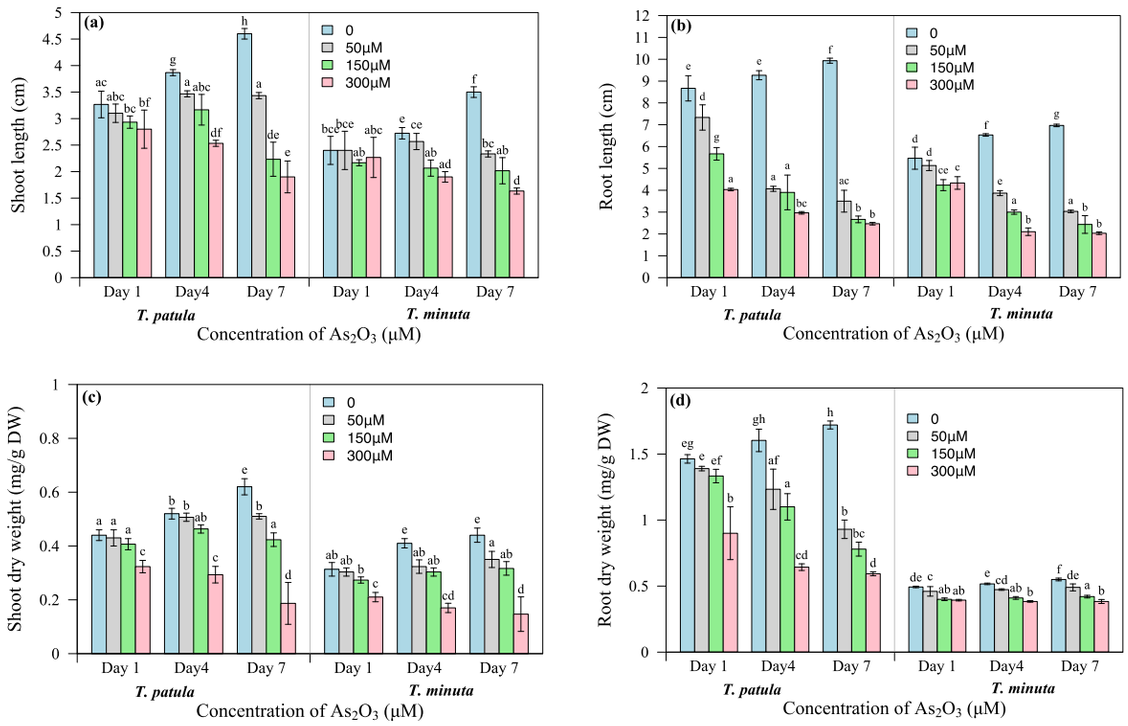

FIGURE 1. Effect of different As_2O_3 treatments on tissue length (a), root length (b), shoot dry biomass (c) root dry biomass (d) of *T. patula* and *T. minuta* for 1-, 4- and 7-days intervals of growth in hydroponic culture. Means of treatment (n = 3) and bars represent standard errors (SE) with the same species and different letters indicate significant differences between arsenic concentrations according to Duncan's new multiple range test

EFFECT OF ARSENIC STRESS ON TISSUE WATER CONTENTS AND TOLERANCE INDEX

A gradual decreasing trend in the tissue water contents was noticed for both species (Figure 2(a)). The decline in tissue water contents was recorded about 3-10% at 50 µM As stress for both species whereas, at 300 µM As stress decrease in water contents was ranged from 18-58% for both species, as compared to the water contents at their control plants (Figure 2(a)) from day 1 to day 7. The tolerance index of both species decreased 20 and 30% at 50 and 150 µM, respectively (Figure 2(b)) on day 7. In addition, on day 4, significantly ($p \leq 0.01$) optimum decrease in tolerance index was more pronounced in *T. minuta* (20-58%) in comparison to *T. patula* (02-43%) at 50 and 150 µM and a similar effect were observed in both species on day 1. However, at the highest As stress level (300 µM), there was a sharp reduction in the tolerance index (65-70%) of both species (Figure 2(b)) on day 7.

EFFECT OF ARSENIC ON CHLOROPHYLL CONTENTS

Total chlorophyll contents in the leaves of both species were also measured (Figure 3). The decreasing photosynthetic content was observed on increasing As exposure in all treatments. The maximum reduction percentage recorded at 50, 150 and 300 µM As which was 53, 66, 76%, respectively, for *T. minuta* in comparison with the control (Figure 3) on day 7 and similar reduction (50, 62 and 75%) in total chlorophyll content was recorded on day 4 at all treatments. A comparison of all treatments showed that *T. patula* maintained comparatively higher chlorophyll content following As exposure. The reduction of total chlorophyll content at the highest As stress level (300 µM) was 57% in *T. patula*, as compared to control on day 7 (Figure 3).

ARSENIC CONTENT IN PLANT TISSUES UPON ARSENIC STRESS

The concentration of As in the shoot and root of both species were also measured with increasing concentration of As and three-time intervals (Figure 4). The As was not detected in control of both plant species while accumulation of As in shoots and roots of treated plants increased with increasing As concentration (Figure 4(a), 4(b)). The accumulation of As pattern was consistent in both species. However, shoots of *T. patula* and *T. minuta* accumulated higher As concentration as compared to roots. The order of As accumulation was roots < stem < leaves. Based on the translocation factor, *T. patula* showed more potential for As translocation from root to shoot. The shoots of *T. patula* accumulated a significantly ($p \leq 0.01$) higher concentration of As (634 µg g^{-1} DW) whereas, *T. minuta* exhibited low concentration (397 µg g^{-1} DW), at 300 µM As treatment on maximum exposure of 7 days (Figure 4(a)). In addition, accumulation of As in the root of *T. minuta* and translocation in the shoot was less in comparison with *T. patula*. Similarly in roots, *T. patula* and *T. minuta* accumulated 482 µg g^{-1} DW and 376 µg g^{-1} DW of As at 300 µM As stress level, respectively, on day 7 (Figure 4(b)).

TRANSLOCATION AND BIOACCUMULATION PATTERN FOR ARSENIC

The phytoextraction ability of *Tagetes* species against As was evaluated by calculating the bioaccumulation factor (BF) and translocation factor (TF) at all As concentrations (Figure 5(a), 5(b)). The highest calculated BF values for *T. patula* and *T. minuta* were 4.3 and 2.4, respectively, at 150 µM of As concentration. However, BF values decreased to 2.7 and 1.7, respectively, at 300 µM for both species (Figure 5(a), 5(b)). The calculated TF values for both species were equal to 1 and *T. patula* showed highest TF (1.3) values at 300 µM of As (Figure 5(a)). For hyperaccumulation, both factors should be above the reference value (1.0). This might explain why *T. patula* has higher biomass production and no toxicity symptoms as compared to *T. minuta* at higher As concentration (300 µM).

DATA SCATTERING UPON EXPOSURE TO THE ARSENIC

The plants were exposed to As, the scatter plot created following PCA demonstrated the difference in the pattern of variables dispersing, indicating that a few parameters were influenced substantially more than the others. The As exposure to *T. patula* resulted in the extraction of three components heaving eigenvalue higher than 1, with the cumulative variance of 94.61%, for the three principal components axis. Total variance of 70.05% was observed for Component 1. Similarly, component 1 had higher loading values for root length, root dry weight, tolerance index, TWC, and total chlorophyll (Figure 6(A); Supplementary Table 1). According to the PCA, Component 2 contributed 12.95% to data dispersion, with higher loading values in Component 2 for shoot length, shoot dry weight, As in shoot, As in root (Figure 6(A); Supplementary Tables 1 & 2). The overall variation between Components 1 and 2 is 82.99%. With eigenvalues larger than 1,

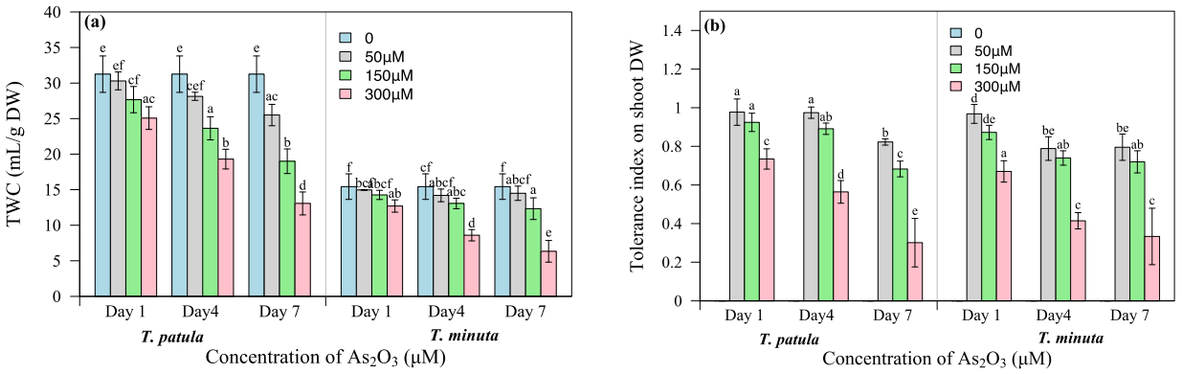

FIGURE 2. Effect of different As_2O_3 treatments on tissue water content (a) and Tolerance index (b) of *T. patula* and *T. minuta* for 1-, 4- and 7-days intervals of growth in hydroponic culture. Means of treatment (n = 3) and bars represent standard errors (SE) with the same species and different letters indicate significant differences between arsenic concentrations according to Duncan's new multiple range test

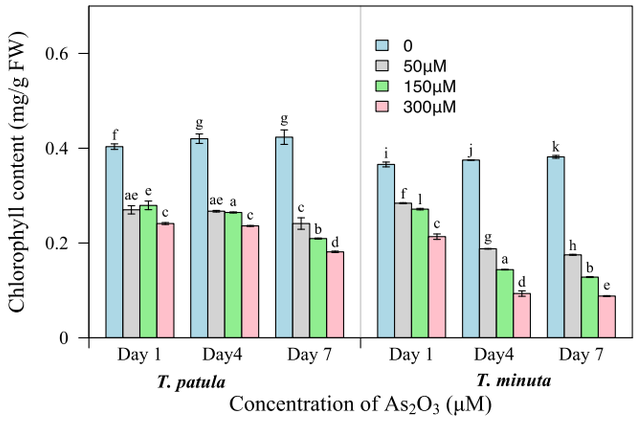

FIGURE 3. Effect of different As_2O_3 treatments on total chlorophyll content in the leaves of *T. patula* and *T. minuta* for 1-, 4- and 7-days intervals of growth in hydroponic culture. Means of treatment (n = 3) and bars represent standard errors (SE) with the same species and different letters indicate significant differences between arsenic concentrations according to Duncan's new multiple range test

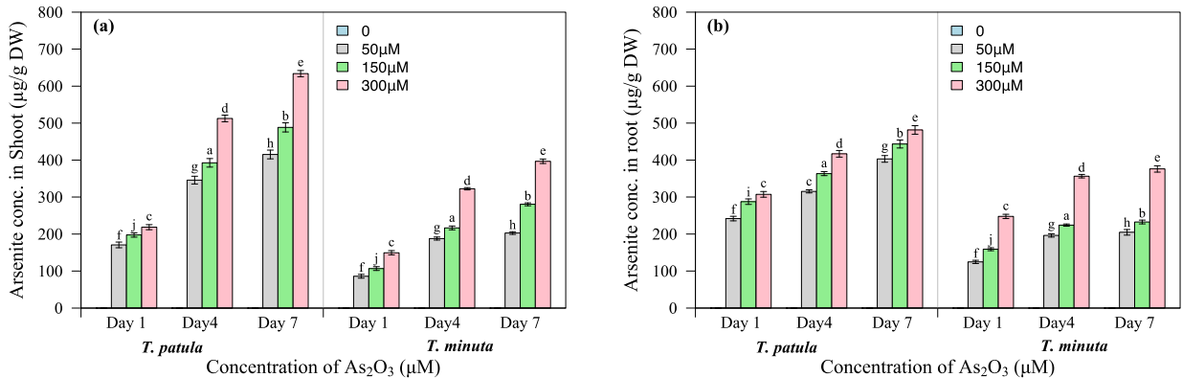

FIGURE 4. As concentration in shoot (a) and root (b) of *T. patula* and *T. minuta* for 1-, 4- and 7- days intervals of growth in hydroponic culture with different As_2O_3 treatments. Means of treatment (n = 3) and bars represent standard errors (SE) with the same species and different letters indicate significant differences between arsenic concentrations according to Duncan's new multiple range test

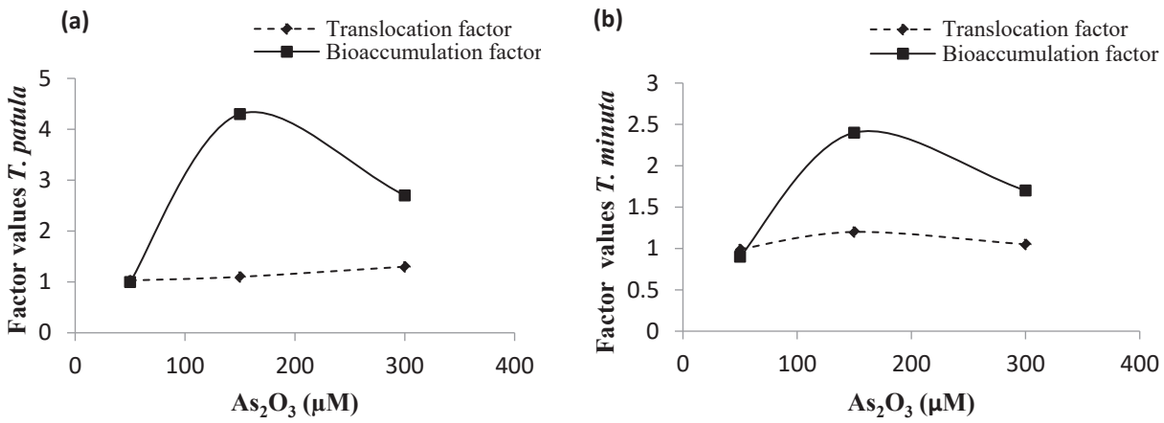

FIGURE 5. Relative translocation factor and bioaccumulation factor of *T. patula* (a) and *T. minuta* (b) at different As concentrations

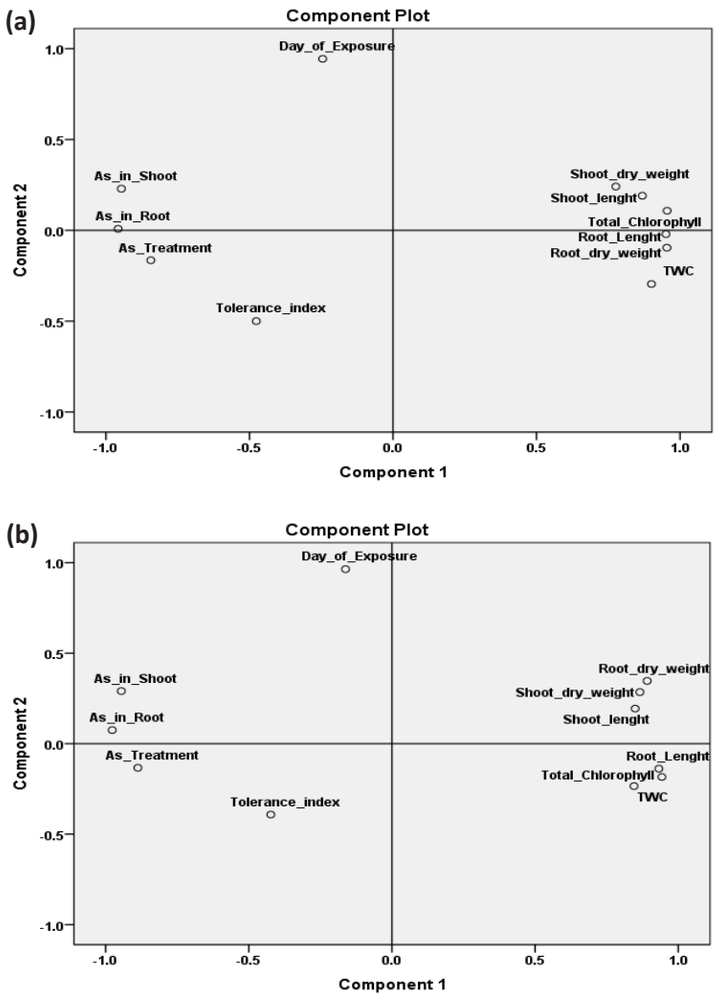

FIGURE 6. Principle component analysis (PCA) plots for the studied parameters upon As exposure with reference to (a) *T. patula*, and (b) *T. minuta*. In PC plots, numerical values followed by PC indicate the percentage contribution in the variability

another component was discovered, representing an extra 11.62% of data variability. Component 1 of the As exposure to *T. minuta* exhibited a total variance of 68.92%, with greater loading values in this component for root length, TWC, total chlorophyll, and tolerance index (Figure 6(b); Supplementary Tables 3 & 4). The component had a total variation of 13.99%, with higher loading values in the component 2 for shoot length, shot dry weight, root dry weight, and As in shoot, As in root (Figure 6(b); Supplementary Tables 3 & 4). These two extracted components have a total variance of 82.91%, indicating that they account for more than 82% of data variability. However, one more component was recovered with eigenvalues greater than 1, showing an additional 10.92% of data variability.

DISCUSSION

Arsenic is a non-essential toxic element and is considered a global pollutant. It is introduced into the environment through natural and anthropogenic processes. Currently, several techniques are available to overcome As contaminates but phytoextraction, using wild plant species, is an environmentally sound, socially accepted and cost-effective method for the removal of As from soil and water (Behera et al. 2014; Elisa et al. 2020; Li et al. 2016; Pietrini et al. 2020). Although limited studies have been documented regarding the exploration of hybrid *Tagetes* (nugget *Tagetes*; a triploid hybrid of *T. erecta* and *T. patula*) (Chintakovid et al. 2007), however, the wild *Tagetes* species like *T. patula* and *T. minuta* which are indigenous to Pakistan, have never been inquired earlier under the hydroponic system with morphological, biochemical, and physiological parameters against As contamination.

The results from this study showed that wild *T. patula* is much more As tolerant and accumulates higher As concentrations in the shoot, than *T. minuta*. This affirmation is based on the analysis of several well-established physiological markers for As toxicity adopted in many previous studies which include; percent seed germination, biomass productivity (Armendariz et al. 2016; Banerjee et al. 2021), water relations, tolerance index and chlorophyll concentration (Abbas et al. 2018; Angulo-Bejarano et al. 2021; Anjum et al. 2017; Sghaier et al. 2015). The plant response at seed germination and early seedling for vegetative growth is important in understanding the survival and tolerance mechanisms under heavy metal stress conditions (Ahmad et al. 2012). Seed germination is considered a crucial feature for seedling establishment and ultimately successful plant growth. Yet surprisingly, percent seed germination of *T. minuta* was significantly ($p \leq 0.01$) higher at low (50 µM) and moderately high As (150 µM) concentrations. However, the chlorotic pattern of leaves and the shoot water contents of the *T. minuta* were considerably affected by As stress whereas, *T. patula* was hardly affected. As is a non-essential element which at higher concentrations may interact with various physiological and biochemical processes resulting in the modifications of plant growth and biomass production (Lin et al. 2008; Pietrini et al. 2020). Biomass production is a significant parameter that can be used to evaluate the ability of plants to tolerate heavy metals in solutions. In this report, a significant ($p \leq 0.01$) reduction was observed in root biomass of treated plants in comparison with the control, while shoot biomass was not affected significantly in both species (Figure 1(c)). Each of As concentrations applied to both species of wild *Tagetes* had damaging effects on the development of plant growth. The reported results are following previous studies on other high tolerance plant species such as chinese bark (Antenozio et al. 2021; Dushenko et al. 1995), rice (Ahmad et al. 2012; Sahoo & Kim 2013) purple willow (Yanitch et al. 2017) and broadleaf cattail (Lyubenova et al. 2013). It has been observed that a higher concentration of As and its prolong exposure hampered the normal life cycle of different plant species.

Root length was reduced drastically in *Tagetes* upon exposure to As stress (50 µM), however, on increasing As concentration both species maintained their root length (Figure 1(b)). Overall, both species showed decreased root dry biomass with As exposure, but the reduction of root dry biomass was higher in *T. patula* (65%), while 31% in *T. minuta*, as compared to control at 300 µM As concentration (Figure 1(d)). The reduction of root biomass following metal treatment represents a typical toxic symptom induced in plants by As (Pietrini et al. 2020; Singh et al. 2007). Changes occurred in the fresh shoot, root length and their dry weights of both plants under treatments of As showed that it directly affected total chlorophyll contents and rate of photosynthesis of plant (Pietrini et al. 2020). Heavy metal ions developed complexes with chlorophyll which results in decreased –SH group activity and ultimately reduced chlorophyll biosynthesis. Similarly, induction of metal stress results in chlorosis of leaves due to a decline in photosynthetic pigments. However, the self-replicating properties of plastids make them more resistant to heavy metal stresses

(Rekik et al. 2017). Consequently, the results in this study proved that *T. patula* maintained comparatively high chlorophyll content on all As exposure.

Generally, plants can use their developed mechanisms such as heavy metals transportation through internal tolerance or cascades of changes through receptors to tolerate heavy metals and maintained cell structure (Reichman et al. 2002). Tolerance index is a good tool to analyse the relationship between growth and the tolerance potential of any plant (Zvobgo et al. 2018). The tolerance indices for different As stress levels were also calculated for both species, which were reduced with an increase in As stress levels (Figure 2(b)) and results were following previous studies (Atabaki et al. 2020; Hong et al. 2011). Similarly, the tolerance of plants decreased with the increasing concentration of heavy metals, and it has been shown that plants with more than 60% tolerance indices were believed to be good tolerant bioreactors (Bianconi et al. 2013). The results exhibited good tolerance to As by *T. patlua* exposed to maximum concentration at 300 µM after four days. Nevertheless, the tolerance index value of both *Tagetes* species indicated that the plant also had good tolerance (70%) to As after being exposed to 150 µM over 7 days.

The hyperaccumulator plants have the capability to accumulate higher metal concentrations and tolerate it without or very few symptoms of stress (Danh et al. 2014; Souri et al. 2017; Wiszniewska 2021) and such plants are most effective for phytoremediation purposes. One major issue in phytoextraction is to select suitable heavy metals accumulators to remove pollutants from a contaminated substrate (Danh et al. 2014; Jiang et al. 2015). The basic four features are the characteristics of metal hyperaccumulators. Firstly, the level of metal concentration ranged equal or above 1,000 µg g^1 dry mass for As, Ni, Co, Cu, and Pb in shoots of a hyperaccumulator (Antenozio et al. 2021; Baker et al. 1989). Secondly, metal translocation property; concentrations of metal in shoots should be higher than roots of plants (concentration in shoots/roots > 1) (Coakley et al. 2019; Dobslaw et al. 2021), the translocation factors of our results are following a higher concentration of metal in shoots. Thirdly, the enrichment factor evaluation (concentration in plant/habitat > 1) (Antenozio et al. 2021; Kumar et al. 2015). Lastly, tolerance property, a hyperaccumulator would have high tolerance toward toxic contaminants (Souri et al. 2017). Both species of *Tagetes* were able to absorb and translocate the As towards their shoots (Figure 5). Particularly, *T. patula* was highly tolerated to As concentration (up to 634 µg g^{-1}) without the appearance of toxicity symptoms on leaves, whereas, in the case of *T. minuta,* most of As was accumulated in roots and could not tolerate up to 397 µg g^{-1} (Figure 4) because of the appearance of toxicity symptoms (chlorosis) on the leaves. In addition, BF and TF were above the reference value (1.0) for both *Tagetes* species. The BF and TF are the common indices used to evaluate plant capacity to pump heavy metals from the substrate (Dobslaw et al. 2021). The comparison of *T. patula* and *T. minuta* showed that *T. patula* exhibited a higher aptitude for bioaccumulation and translocation of As towards its shoot.

Based on plant growth parameters, *T. minuta* is more sensitive at high As concentration (300 µM) in comparison to *T. patula*. The *T. patula* was found to be capable of transferring efficiently the absorbed metals towards its shoot which is reflecting the higher values of its TF. Additionally, total As (As contents in root and shoot) removed from the hydroponic was also significantly high for *T. patula* as compared to *T. minuta. T. patula* showed the ability to absorb and accumulate much higher concentrations (634 µg g^{-1}) of As in the shoots as compared to the roots. This showed that *T. patula* is a positive indicator with the potential capability to serve as a phytoremediator. The PCA analysis of the investigated parameters of *T. patula* and *T. minuta* to As exposure revealed the plant's adaptability to stress (Figure 6). Consequently, the current work established a better understanding of As accumulation and tolerance mechanisms in *T. patula* and *T. minuta*. This study also provides a notion regarding the variation which exists between the species of the same genus for As accumulation and tolerance and has been well established for other metals (Madanan et al. 2021; Nawaz et al. 2017). However, further exploitation of such variations may provide scientists with a better candidate for phytoremediation from genus *Tagetes*. Therefore, the present study gives valuable evidence and data supporting wild *Tagetes* species (*T. patula*) for phytoremediation purposes. Further research is required to obtain information about the mechanism of As uptake, transport and efflux along with the metabolic basis and identify/characterizing the genes involved which may prove helpful to improve the tolerance and accumulation ability of this plant species.

Conclusions

This study showed that the bioaccumulation of As metalloid and capacity to tolerate its toxic effects varied between both plants and their organs. However, *T. patula* can activate the physiological system more to

tolerate and hyperaccumulate As elevated concentration in hydroponics. Particularly, growth of the plants can preserve their physiological status even though some adverse effect was observed on chlorophyll at high dose treatment. Notably, the tolerance for As metal and the potential of *Tagetes* species for growth can provide useful indication and suitability of this plant for phytoremediation.

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*Corresponding author; email: irumayaz@cuiatd.edu.pk

SUPPLEMENTARY TABLE 1. Contribution of extracted components in total data variance with *T. patula*

Component	Initial Eigenvalues		
	Total	Variance (%)	Cumulative (%)
1	7.705	70.045	70.045
2	1.424	12.946	82.991
3	1.279	11.628	94.619
4	0.250*	2.269	96.888

Extraction Method: Principal Component Analysis.
*as the Eigen value of principle component 4 (PC 4), was less than 1 so no further PC were presented

SUPPLEMENTARY TABLE 2. Component score/PCA loading values of studied traits in extracted components *T. patula*

Traits	Component	
	1	2
Shoot_length	.113	.134
Shoot_dry_weight	.101	.169
Root_length	.123	-.014
Root_dry_weight	.124	-.067
TWC	.117	-.208
Tolerance_index	-.062	-.351
Total_chlorophyll	.124	.076
As_in_shoot	-.123	.161
As_in_root	-.124	.006
Day_of_exposure	-.032	.663
As_treatment	-.109	-.116

Extraction Method: Principal Component Analysis.

SUPPLEMENTARY TABLE 3. Contribution of extracted components in total data variance with *T. minuta*

Component	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
1	7.581	68.916	68.916
2	1.539	13.990	82.905
3	1.114	10.124	93.029
4	0.356*	3.236	96.266

Extraction Method: Principal Component Analysis.
*as the Eigen value of principle component 4 (PC 4), was less than 1 so no further PC were presented

SUPPLEMENTARY TABLE 4. Component score/PCA loading values of studied traits in extracted components *T. minuta*

Trait	Component	
	1	2
Shoot_length	.112	.126
Shoot_dry_weight	.114	.185
Root_length	.123	-.090
Root_dry_weight	.118	.225
TWC	.112	-.152
Tolerance_index	-.056	-.255
Total_chlorophyll	.124	-.120
As_in_shoot	-.125	.189
As_in_root	-.129	.049
Day_of_exposure	-.021	.627
As_treatment	-.117	-.087

Extraction Method: Principal Component Analysis.