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Available online at www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2013, 5(4):431-437

Notulae Scientia Biologicae

Anatomical Response of *Amaranthus hybridus* Linn. as Influenced by Pharmaceutical Effluents

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Abstract

Anatomical studies were carried out on the leaves, stems and roots of *Amaranthus hybridus* subjected to irrigation of 10%, 20%, 30% and 40% concentrations of pharmaceutical effluents to identify the responses of this plant to the treatment. Leaf structures of *A. hybridus* showed no significant change due to the effect of the effluents. Significant reduction was observed in the trichome density and number of epidermal cells at the adaxial surface as from the 20% effluent concentration upward ($p<0.05$). Stomatal size significantly reduced on both leaf surfaces as from 20% effluent concentration upward while stomatal density increased significantly at the adaxial surface from the 20% effluent concentration ($p<0.05$). Tetracytic and anisocytic stomatal complex-types were observed in *A. hybridus* with varied modifications from 20% effluent concentration upward at both leaf surfaces. Vessels and phloem cells in the stems and roots of *A. hybridus* were seriously affected by the effluent. Vessel walls were thickened and their width reduced significantly as from 20% effluent concentration upward while phloem cells experienced progressive loss of structural integrity from the same concentration (20%) upward in both the stems and roots. This study showed that the pharmaceutical effluents have toxic effect on *A. hybridus* and the effects were more pronounced as from 20% concentration.

Keywords: irrigation, parenchyma cells, pharmaceutical effluent, phloem cells, vegetables, vessel walls

Introduction

The use of industrial wastewater/effluents in the process of irrigation of food crops especially leafy and fruit vegetables has increased in recent years in urban and peri-urban areas due to shortage of clean water (Arora *et al.*, 2008). The use has also been promoted by urban farmers due to the belief that such industrial effluents contain high nutrients that can promote rapid vegetative growth of their crops, reduce or eliminate the cost of fertilization either in organic or inorganic form (Swamenathan and Vaidheeswaran, 1991; Ahmad *et al.*, 2006). The use of industrial effluents could be a technical solution to reducing soil degradation through chemical pollution of soil (Fatoba *et al.*, 2011) without considering the detrimental effects the chemical constituents of such effluents on the physiological processes, morphological and anatomical structures of such crops irrigated with the effluent (Wyszkowski and Wyszkowska, 2003; Kovacic and Nikolic, 2005).

In Nigeria, most of the urban farmers divert effluents (either known to be treated or untreated) to farmlands to irrigate their vegetable farms to meet up with the rising demand for fresh vegetables (Uaboi-Egbenni *et al.*, 2009; Fatoba *et al.*, 2011). Reports have it that in Nigeria, vegetables are produced throughout the year e.g. Fatoba *et al.* (2011) due to the availability of industrial effluent to irrigate. However, there should be cautious use of effluents for irrigation of crops that are tender and herbaceous like vegetables. Kumar and Bhargava (1998) cautioned on the deleterious effects of higher concentrations of effluent. Dutta and Bosgya (1997) using paper mill and tannery effluent observed decreased chlorophyll content in *Lycopersicon esculentum*. Karunyal *et al.* (1993), Somasheka and Siddaramaiah (1997) (using soap and detergent effluent) reported the germination of *Pennissetum tyhoides*, *Pisum savitum* and rice seeds were suppresssed at various high effluent concentrations.

Pharmaceutical effluents are wastes generated by pharmaceutical industry during the process of drugs production. Some pharmaceutical effluents are known to contain high concentrations of organic compounds and total solids, mercury, cadmium, isomers of hexachlorocylohexane, 1, 2-dichloroethane and solvent. The biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids as well as phenol and pH of pharmaceutical effluents are however not consistent, all these depends on the product manufactured, materials used and the processing details (Anonymous, 1993). The production of bulk drugs has recently been identified as an important source of environmental pollution with active pharmaceutical ingredients (APIs) at certain locations (Larsson *et al.*, 2007; Li *et al.*, 2008; Fick *et al.,* 2009).

Received 04 July 2013; accepted 07 November 2013

A. hybridus, commonly called smooth amaranth, smooth pigweed, red amaranth, or slim amaranth, is a species of annual flowering plant. *A. hybridus* grows from a short taproot and can be up to 2.5 m in height. In Nigeria, *A. hybridus* leaves combined with condiments are used to prepare soup (Oke, 1983; Mepha *et al.*, 2007). *A. hybridus* has been shown to contain large amount of squalene, a compound that has both health and industrial benefits (Smith, 2000; He and Corke, 2003). Due to high demand for this vegetable in Nigeria, urban farming of *A. hybridus* through effluent irrigation has grown tremendously. Several researchers have reported the effects of such effluents irrigation on the nutrient and heavy metal contents of vegetables (Abdullahi *et al.*, 2007: Arora *et al*., 2009; Gupta *et al.,* 2008; Fatoba *et al.*, 2012; Mapanda *et al.*, 2005; Nwachukwu and Mabagwa, 2008; Srivastava, 1991; Wang *et al.*, 1991). This research work was designed to assess structural changes of *A. hybridus* in response to the irrigation pharmaceutical effluents.

Materials and methods

Experimental design

Pot experiment was conducted at the Screen house of the Department of Plant Biology, University of Ilorin, north-central of Nigeria to assess the effects of pharmaceutical effluents on anatomical structures of the leaves, stems and roots of *Amaranthus hybridus* L (Amaranthaceae). Topsoil obtained from the experimental farm of the University was sieved to remove gravels and debris with a 2 mm mesh. A sample of 2.5 kg of soil was filled into each of the 15 white plastic containers and arranged in completely randomized block design.

Seeds of *A. hybridus* were planted in containers tagged Control, 10%, 20%, 30% and 40% respectively as treatment with 3 replicates each. Seedlings were thinned to four plants per container after two weeks of germination. Pharmaceutical effluents collected at the disposal channel of Afrab-Chem Limited, Lagos, southwest Nigeria was serially diluted with borehole water to give representative concentrations of 10%, 20%, 30% and 40%.

Normal bore hole water irrigation of *A. hybridus* was carried out until the first round of the irrigation with 50 ml each of the prepared concentrations of the effluents was carried out at the fourth week after planting (4WAP). Effluents irrigation of the vegetables was also carried at 6WAP and normal borehole irrigation commenced till the 8WAP when the experiment was terminated. Samples of the leaves, shoots and roots were collected after the eight weeks of growth for anatomical studies and heavy metal analysis.

Isolation of leaf epidermal layer

Three samples of the leaves of *A. hybridus* from each of the treatments were collected and prepared as specimens for anatomical study. Leaf segment of an area of1cm^{2}from each specimen was macerated according to the method of Alvin and Boulter (1974) as amended by Ogunkunle *et al.* (2013). The upper (adaxial) and the lower (abaxial) surfaces were separated with dissecting needle and forceps and rinsed with clean water. Each specimen was stained with 1% aqueous safranin for 20 min and rinsed in water (method modified by Olofinobinu and Oladele (1997). The samples were then mounted on glycerine jelly for microscopic observation using a Olympus research microscope fixed with an Amscope camera (FM A050).The epidermal cell, anticlinal cell wall, stomatal size, density and stomatal complex-types were studied using the methods of Franco (1939), Salisbury (1927), Stace (1965), and Weyers and Meidner (1990). Terminologies for naming stomatal complex types followed those of Dilcher (1974), Metcalfe and Chalk (1988) and of Weyers and Meidner (1990).

Transverse sections of stem and root

Thin free hand sectioning of the stem and root were made using a sharp razor blade, stained with aniline blue and mounted on glycerine for microscopic observation. Number of vascular bundles, vessels, phloem and parenchyma cells were counted and their sizes measured with ocular micrometer. Photomicrographs of the sections were taken with Celestron-Pentaview LCD Digital Microscope (Model No. 44348).

Statistical analysis

Mean of all the parameters were subjected to Analysis of Variance (ANOVA) and means separated by Duncan Multiple Range Test at $p<0.05$. All statistics were carried out with the use of Statistical Package for Social Sciences (SPSS) version 16 and Origin 7 software.

Results

Variation in the epidermal features of the leaves of A. hybridus

There was presence of multicellular trichomes and stomata (anisocytic and tetracytic) on both (adaxial and abaxial) surfaces of the leaf epidermis of *A. hybridus* (Fig. 1).

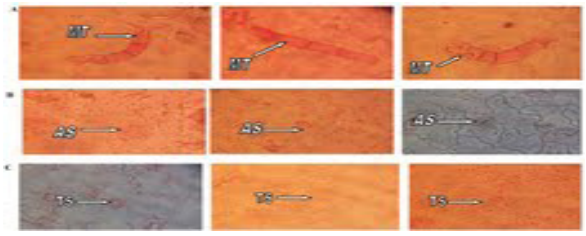

Fig. 1. Leaf epidermal features of *A. hybridus* showing (A) multicellular trichome (MT), (B) anisocytic stomata (AS) and (C) tetracytic stomata (TS). X400

There was the presence of 'wavy' anticlinal wall at the abaxial leaf surface in all the treatments (Control, 10%, 20%, 30% and 40% effluents) while 'straight' and 'curvy, anticlinal walls were observed in the adaxial surface of the leaves in all the treatments (Tab. 1).

Tab. 1. Epidermal features of leaves of *A. hybridus* irrigated with various concentrations of pharmaceutical effluents

Parameter	Leaf surface	Control	10%	20%	30%	40%
Anticlinal wall	Abaxial Adaxial	Wavy Straight Curvy	Wavy Straight Curvy	Wavy Straight Curvy	Wavy Straight Curvy	Wavy Straight Curvy
Trichome type	Abaxial Adaxial	Multicellular Multicellular	Multicellular Multicellular	Multicellular Multicellular	Multicellular Multicellular	Multicellular Multicellular
Trichome density (per mm^2)	Abaxial Adaxial	1.7^{a}± 0.58 2.7^{a}± 0.96	1.3^{a}± 0.58 3.2^{a}±1.79	1.7^{a}±0.58 2.0^{ab}±1.00	1.7^{a}±0.58 1.4^{b}±0.55	2.3^{a}±0.58 1.3^{b}±0.58
No of epidermal cells	Abaxial Adaxial	20.5^{b}±2.75 37.8^{a}±4.15	25.8^{ab}±4.60 33.5^{ab}±5.24	31.2^{a}±6.50 30.2^{b}±3.50	30.0^{a}± 3.08 29.4^{b}±2.60	30.8^{a}±2.68 28.8^{b}± 4.21

Values with the same superscripts are not statistically different at p<0.05

But this shows that the effluent had no effect on anticlinal walls of abaxial and adaxial surfaces of the leaves. Density of multicellular trichome at the abaxial surfaces was not significantly different in all the treatments except the 40% concentration while density of trichomes at the adaxial surface was significantly higher in the Control and 10% treatments and reduced in the 20%, 30% and 40% effluent treatments (p<0.05). The number of epidermal cells in the abaxial surface of 20% and higher concentrations were statistically more than the control and 10% treatment but the reverse was the case for the adaxial surface except for the 10% treatment that was statistically the same with the control (p<0.05). Fig. 2 shows the differences in trichome density and number of epidermal cells between abaxial and adaxial surfaces.

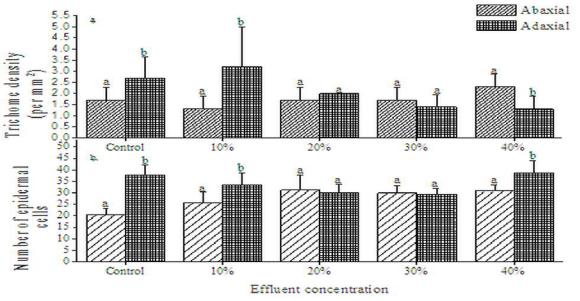

Fig. 2. Epidermal leaf structures of *A. hybridus* irrigated with pharmaceutical effluent; (A) trichome density and (B) number of epidermal cells

Trichome density differed significantly between the two surfaces in the Control, 10% and 40% treatments while no significant difference was observed in 20% and 30% effluent treatments at p<0.05 (Fig. 2a). The same pattern was also observed for the number of epidermal cells across the five treatment regimes between the abaxial and adaxial surfaces (Fig. 2b). These observations showed that the effluent affected the trichome density and number of epidermal cells of the leaves.

Stomatal size in the abaxial surface of the leaves of *A. hybridus* was affected by the effluent such that significant reductions were observed as from the 10% effluent treatment (p<0.05).There seems to be no significant different in the stomatal size of the adaxial surface of the leaves (p<0.05) (Tab. 2). Stomatal density of the abaxial surface recorded no significant difference while observed significant difference was recorded in the adaxial surface of the treatments. Stomatal density was significantly the same in the Control and 10% effluent treatments and significantly increased in the 20% and 30% effluent treatments but reduced significantly at 40% treatments (p<0.05) (Tab. 2). The two types of stomatal complex-types observed in A. hybridus were tetracytic and anisocytic. Frequency of tetracytic stomatal complex-type were the same in the Control and 10% effluent treatments but significantly reduced in 20% effluent treatments and recorded the same number with 30% and 40% treatments (p<0.05). A different twist was observed at the adaxial surface; the least number of tetracytic complex-type was observed in the Control and 40% effluent treatments while 20% effluent treatment had the highest occurrence of tetracytic complex-type at the adaxial surface (Tab. 2). There was no significant difference in the frequency of anisocytic complex-type at the abaxial surface while significant variations were observed at the adaxial surface. Twenty percent (20%) effluent treatment had the greatest number of anisocytic complex-type at the adaxial surface while the Control recorded the least at p<0.05 (Tab. 2).

Tab. 2. Effects of pharmaceutical effluents on stomatal features of *A. hybridus*

Parameter	Leaf surface	Control	10%	20%	30%	40%
Stomatal size (µm2)	Abaxial Adaxial	37.1^{a}±8.24 30.0^{a}±5.95	28.1^{b}±6.18 27.4^{ab}±3.69	26.8^{b}±4.30 24.6^{b}±2.77	28.3^{b}±5.64 28.5^{ab}±4.34	25^{b}±5.06 26.2^{ab}±2.22
Stomatal density (per mm2)	Abaxial Adaxial	26.0^{a}±4.40 10.0^{b}±0.82	26.7^{a}±3.59 12.7b±3.45	20.6^{a}±4.20 25.3^{a}±4.08	24.0^{a}±5.57 23.8^{a}±3.67	22.3^{a}±4.46 11.0^{b}±7.32
Frequency of stomatal complex-type (%)						
Tetracytic	Abaxial Adaxial	19.7^{a}±2.93 5.4^{d}±1.72	21.0^{a}±5.89 7.7^{b}±1.67	14.1^{b}±2.34 11.8^{a}±1.17	17.0^{b}±3.70 15.8^{a}±1.77	17.7^{b}±1.38 5.8^{c}±2.17
Anisocytic	Abaxial Adaxial	5.7^{a}±2.76 4.5^{c}±1.38	5.7^{a}±2.75 5.4^{b}±2.15	5.1^{a}±1.73 9.67^{a}±2.16	7.0^{a}±2.61 6.57^{a}±1.27	4.83^{a}±1.33 5.3^{b}±0.76

Values with the same superscripts are not statistically different at p<0.05

Anatomical structures of the roots and stems of A. hybridus due to the pharmaceutical effluents irrigation

Changes in the anatomical structures of the roots and stems of *A. hybridus* observed in the various effluent concentration treatments are presented in Figs. 3, 4 and 5. Effluent treatment of 20% increased the number of vascular bundles in the roots significantly while 30% and 40% treatments reduced the number of vascular bundles significantly at $p<0.05$ (Fig. 3a). Multiplication of parenchyma cells was initiated significantly in the 10% and 20% effluent treatments whereas 30% and 40% showed no effect on the number of parenchyma cells ($p<0.05$) (Fig. 3a).

The stems showed a significant decreasing trend in the number of vascular bundles from the 20% effluent treatment and the least number of vascular bundles was recorded in the 40% effluent treatment at $p<0.05$ (Fig. 3b). However, a different pattern was observed in the number of parenchyma cells; all the effluent treatments recorded statistically the same number of parenchyma cells at $p<0.05$ except the 40% effluent treatment that recorded significant higher number of parenchyma cells ($p<0.05$).

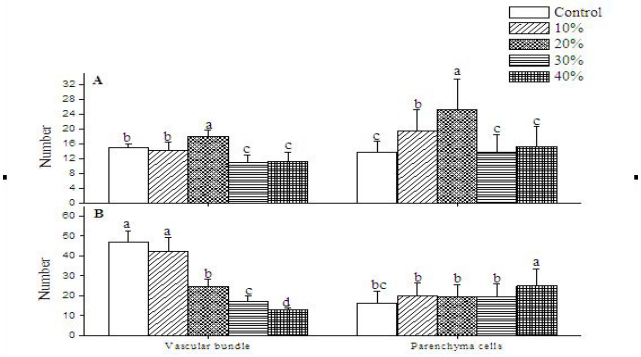

Fig. 3. Variation in the effects of various concentrations of pharmaceutical effluents on tissue components of (a) root and (b) stem of *A. hybridus*

Effects of the effluent treatments on vessels and phloem cells of the roots and stems of A. hybridus

Vessels of the roots were observed to show structural integrity with no pathological condition in the Control and 10% treatments and the phloem were intact with numerous cells (Fig. 4a, b and c). Progressive vessel wall thickening was observed from 20% effluent concentration to higher concentrations (Fig. 4d). Vessel walls were thickened in the 30% and 40% effluent treatments with death of some vessel members making the number of vessel members to reduce and there was the loss structural integrity of the phloem cells which eventually made the phloem cells to collapse (Fig. 4 e and f).

Stems of *A. hybridus* in the Control and 10% effluent treatments showed no observable abnormality in the structural integrity and number of the vessels and the phloem cells (Fig. 5a and b). The regime of 20% effluent treatment showed progressive widening of the vessel walls and deterioration of phloem cells (Fig. 5c). The toxic effects of the effluents were pronounced in the 30% and 40% effluent treatments with significant widening of the vessel walls and pathological death of the phloem cells due to the total collapse of the phloem structural integrity (Fig. 5 d and e).

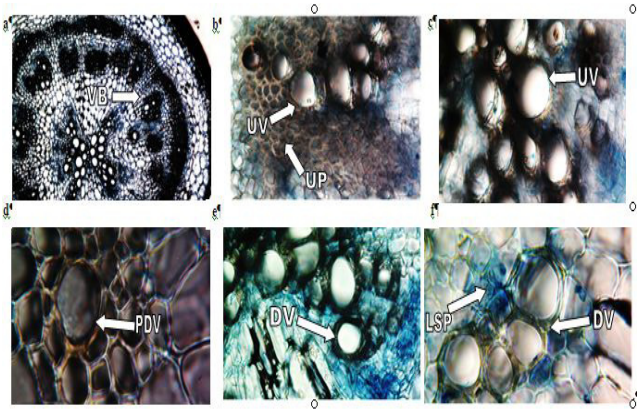

Fig. 4. Transverse section of the root of A. hybridus of the Control (a) showing vascular bundles (VB) with no pathological condition, (b) Control with undamaged vessels (UV) and phloem (intact structural integrity) (UP), (C) 10% treatment with undamaged and numerous vessels(intact structural integrity) (UV), (d) 20% treatment with progressive vessel wall thickening (PDV), (e) 30% and (f) 40% treatments with damaged, thickened and reduced number of vessel walls (DV) and visible loss of structural integrity of the phloem (LSP). X400

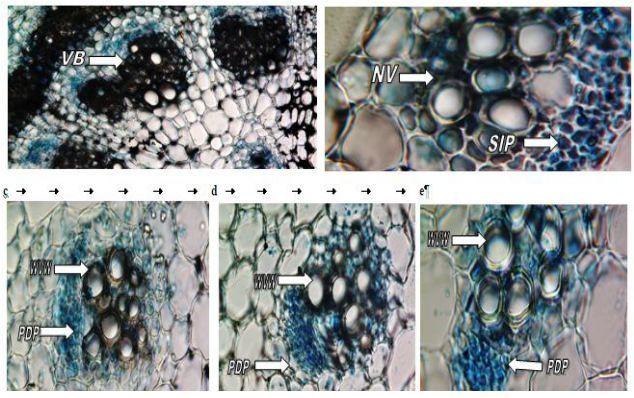

Fig. 5. Transverse section of the stem of A. hybridus of showing (a) the Control with intact vascular bundles, (b) 10% treatment with normal vessels (NV) and structurally-intact phloem (SIP), (c) 20%, (d) 30% and (e) 40% treatments with wide-vessel walls (WVW) and loss of structural integrity and pathological death of the phloem (PDP). X400

Discussion

Effects on the pharmaceutical effluent on leaf epidermal structures of A. hybridus

The use of industrial effluents for irrigation of crops especially leafy and fruit vegetables poses several overt anatomical alterations to the crops. Leaf epidermis of *A. hybridus* showed varied responses to the various concentrations of the pharmaceutical effluents. There was no difference in structures of the anticlinal wall and trichome of the leaf epidermis in all the treatments. This has also been reported by Omosun *et al.* (2008) in *A. hybridus* irrigated with crude oil. The toxic effects of the pharmaceutical effluents were observed from the 20% to 40% effluent concentrations in the form of reduction of the trichome density and number of epidermal cells at the adaxial surface. This perhaps may be a strategy to reduce stress from physiological processes at the adaxial surface since it is the surface receiving much of the solar radiation and engages in photosynthesis more. The abaxial surface experience significant reduction of the epidermal cells from 20% effluent concentration which could only be premised on the level of morphological aberration in the stomatal formation due to the toxic effects of the effluent. This has been previously affirmed by Esau (1965) that the degree of sinuosity of epidermal cells was premised on the extent of impact exerted on the stomata in the course of developmental process.

It is also clear that stomatal density appeared to increase significantly at 20% and 30% concentrations and decreased at 40% concentration. This could be as a result of modification mechanism of *A. hybridus* to survive in the polluted substrate by replenishing dead stomata due to the toxic effects of the effluents. Increased stomatal density is considered as adaptability indicator to a polluted environment (Kapitonova 2002; Gostin 2009; Ogunkunle *et al.*, 2013). This modification method was impossible for the amaranth plants treated with 40% effluent concentrations due to the high toxicity effect of the effluent.

The decreased stomatal size (both leaf surfaces) in all the treatments aside the Control is an indication of A. hybridus survival strategy in the presence of pollutants from the pharmaceutical effluents. Reduced stomatal size helps in increasing the rate of photosynthesis without excessive transpiration according to Melo *et al.* (2007). This also assist in lowering the rate of transpiration and reduce stomatal conductance in the presence of stress of chemical pollutants as observed in the leaves of *Cenchrus cellaris* by Vijayakumar and Udayasoorian (2007). This was also reported by (Abdulrahaman, 2009; Abdulrahaman and Oladele, 2012) in which stomatal size of some ornamental and xerophytic plants were reduced which later translated to reduced rate of transpiration. Noman *et al.* (2012) affirmed that this type stomatal size modification is an indication of the presence of heavy metal toxicity which could prove that the pharmaceutical effluents may have contained toxic heavy metal.

The presence of stomata with large number of subsidiary cells like the tetracytic and anisocytic complex-types observed showed that *A. hybridus* is a plant with great transpiration as Abdulrahaman and Oladele (2007) opined that stomata with multi-subsidiary cells facilitate good gas exchange. Due to the importance of the adaxial surface in photosynthesis and gas exchange, the impact of the toxicity of the effluent was most felt on the leaf surface. The frequency of tetracytic and anisocytic complex-types seemed to increase as from 20% concentration, this amy be a coping strategy. It is obvious that 40% concentration of the effluent might be too toxic for *A. hybridus*, thereby displaying the most deleterious effects of the effluent.

Effects on the pharmaceutical effluent on the roots and stems of A. hybridus

The pharmaceutical effluents showed serious toxic effects on the vascular bundles of the roots and the stems of *A. hybridus* in the form of reduction of the number of vascular bundles present especially in the stem. The reducing effect on the vascular bundles in the stem was first initiated by the lowest concentration whereas toxic effects were only felt by the 30% and 40% effluent treatments. This indicates that roots of *A. hybridus* could be resilient to certain extent to the toxicity of the pharmaceutical effluents. Proliferation of parenchyma cells in the root of *A. hybridus* was promoted at very low concentrations (10%) of the pharmaceutical effluents but its toxic effect resulted in drastic reduction of the number of parenchyma cells at 30% and 40% concentrations. This showed that at low concentrations, *A. hybridus* was able to increase the production of parenchyma cells which constitute an adaptive mechanism to regulate ion concentrations entering the root as reported by Strogonov (1964) in Vijayakumar and Udayasoorian (2007). But at high concentrations which seem to be toxic, the number of parenchyma cells was reduced in order to prevent excess ions from the effluents entering the xylem and reduce toxicity from effluent conduction to the aerial parts. This kind of mechanism was observed by Vijayakumar and Udayasoorian (2007) in which parenchyma cells of *Cenchrus cellaris* was reduced to prevent excess toxic ions from entering the root vessels. Moreover, crude oil application at 1-3% to *A. hybridus* also reduced the number of parenchyma cells in the study by Omosun *et al.* (2008) which they attributed to physiological drought. Different patterns were observed in the parenchyma cells of the stem of *A. hybridus*. Promotion of differentiation of parenchyma cells was initiated, especially at the highest concentration. This could probably be due to the fact that most toxic constituents of the effluents must have been complexed or sequestered before getting to the parenchyma cells. Concentrations above 10% posed deleterious impacts on the vascular bundles of the

roots and stems of *A. hybridus*. It is very clear that at 20% concentration, progressive toxic effects emerged in form of thickening of the vessel walls with reduced width and reduction in the number of the vessels in the roots. This is evident in the roots since the vessels are the first point of attack by the toxic effluents as they are conducted from the rhizosphere. Total collapse or loss of structural integrity of the phloem cells observed in the roots and stems of *A. hybridus* was as a result of the extreme toxicity of the pharmaceutical effluent. Reduced width of vessels in plants irrigated with industrial effluents has already been reported by Khan *et al.* (1984a; 1984b), Mahmood *et al.* (2005), and by Tyagi *et al.* (2012).

Conclusion

Pharmaceutical effluents portend toxic effects on *A. hybridus* in such a way that several anatomical structures were modified as found in the leaf epidermis and conducting elements in the roots and stems were damaged. It is evidently clear from the study that low concentration of this effluent can be non-toxic to vegetable growth but increasing the concentration can be covertly toxic to the vegetable which possibly may translate to low biomass production.

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