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International Journal of Plant Physiology and Biochemistry

Full Length Research Paper

Vegetative propagation of *Echinops giganteus* using stem and root cuttings

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***Echinops giganteus* (Asteraceae) is a shrub native to Cameroon. As part of a recovery effort focused on *Echinops giganteus*, a vegetative propagation technique was developed. Plants were vegetatively propagated in and out of a non-midst propagator, juvenile stem and root cuttings from the nursery and plant growth regulator hormones of concentrations: 0, 0.5, 1, 2, 3, 4, 5, 8 and 10 g/L. The effect of the cutting positions from the mother plant (apical, medial and basal) and concentration of growth hormones (indole butyric acid and naphthaleneacetic acid) on rooting success were evaluated. Cuttings were dipped in into a commercial insecticide commonly called EAGROW before putting it into hormone concentrations; the dilution media for hormones was alcohol. Rooting occurred with and without auxin treatments but was greatest in the control concentrations (just alcohol) for both hormones; rooting was lowest when hormone concentrations were greater than 3 g/L. Rooting success was evaluated two months after the experiments were started. None of the stem cuttings survived. The control root head cuttings in the propagator had the lowest mortality rates than (NAA 11% and IBA 33%). The trails out of the propagator hard a 0% mortality rate. Vegetative propagation of *E. giganteus* will allow its large-scale regeneration for a sustainable management plan.**

Key words: Vegetative propagation, stem and root cuttings, indole-3-butyric acid (IBA), ∝-naphthaleneacetic acid (NAA).

INTRODUCTION

The environmental degradation in tropical world is an inevitable outcome of developmental activities. The ecosystem services are now shrinking due to erosion of genetic biodiversity in natural ecosystem. There is faster harvesting of natural resources due to increasing population and demand for material development. The economic growth depends much on the use of natural resources (Upadhyay et al., 2010).

The genus Echinops is of the Asteraceae family and consist of about 120 species distributed world-wide (Garnatje et al., 2004). *Echinops giganteus* has been designated a non-forest timber product (NTFP) in the Congo Basin and the part exploited is the root (Tchatat, 1999).The roots have diverse uses spanning from medicinal, culinary to industrial (Noumi, 1984; Menut et al., 1997). The root of this plant is used to treat heart and gastric troubles (Tene et al., 2004). The root has aromatic properties and has been collected and distilled to obtain essential oils which are used in synergy with those from other plants to eradicate weevils in stored grains (Ngamo

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et al., 2007; Pérez et al., 2010). This species is also of interest to the fragrance and flavor sectors Menut et al., 1997).

The main problem with *E. giganteus* is that, despite all its importance and its conservation status as a nearly threatened species, no implementation of conservation, management and sustainable use strategies have been put in place, due to lack or insufficient scientific data on their regeneration. This general lack of information is in particular related to lack in Sub- Saharian zone of expertise and infrastructures to carry out propagation experiments. Vegetative propagation is also a practical means for mass production of high quality regeneration stock. Unlike with sexual propagation, the new independent plant produced through vegetative means is a clone in which desirable traits of the donor are preserved (Santoso and Parwata, 2014). Moreover, vegetative propagation can bypass the germination phase to reduce the rotation period of the species. Stem cutting is the most common of vegetative propagation methods for herbaceous and woody plants. Reasons for the popularity generally revolve around the low cost (Waziri et al., 2015) and ease (Dawa et al., 2017) associated with the use of the technique. The success of cutting propagation may be confounded by the age of the donor plant (Ambebe et al., 2017), growth medium (Ashiono et al., 2017), type of cutting (Washa, 2014), phytohormones (Bhardwaj et al., 2017), size of the cutting and health of the donor plant (Kramer and Kozlowski 2014) among others. Furthermore, the responses of the cuttings to some of these factors are species sensitive (Hassanein, 2013). Rooting hormones are very important in the rooting process of cuttings (Wiessman-Ben and Tchoundjeu, 2000). Their beneficiary effect was also confirmed by Aminah et al. (1997), Arya et al. (1994), Al-Saqri and Alderson (1996) and Hartmann et al. (1997). According to Wiessman-Ben and Tchoundjeu (2000) hormones such as auxin (IBA, IAA, NAA) play an important role in root growth, whereas hormones like gibberellins are important in the physiological process of the plants such as in stem elongation and bud development. Among the exogenous rooting hormones, indole-3-butyric acid (IBA) and ∝-naphthaleneacetic acid (NAA) are two synthetic chemicals that have been found to be reliable in root promotion of cuttings. IBA is widely applied for general use because it can remain non-toxic within a wide range of concentrations and improves root initiation of cuttings for most plants species (Al-Barazi and Schwabe, 1982; Hartmann et al., 1997; Hartmann et al., 2002). This research work is therefore designed to come out with the best method for the propagation of *E. giganteus* in the Western Highlands of Cameroon.

MATERIALS AND METHODS

This study was carried out in Menoua, a sub-division fond in the Western Highlands of Cameroon.

Study site

Localization of the Menuoa division

The germination, transplant experiments and part of the floristic inventory was carried out in Dschang situated in the Menuoa Division in the Western Region of Cameroon (Figure 1). It has geographic coordinates, latitude 5° 26'N, longitude 10° 26'E and an altitude 1,400 m. According to the data of the meteorological station of the IRAD of Dschang, there is an equatorial climate characterized by an average annual temperature of 20.1°C and Annual rainfall is 2000 mm on average (Aghofack-Nguemezi and Tatchago, 2010).

The vegetation consists, to a large extent, of savannah grassland, with the Poaceae forming the main vegetation layer, interspersed with a few other annuals, biennials and perennials trees (Ngwa, 1979). According to Aswingnue (2003), the vegetation of this region is both natural and cultivated. The cultivated vegetation consists of planted trees like *Cola accuminata*, Eucalyptus globulus, *Raphia hookeri* and other fruit trees. Eucalyptus globulus lies mostly in the low lying plains, while woody valley and natural forest exist in the watershed area (Tematio et al., 2001). The soil texture is silt-clay-loam which makes it very fertile for agricultural activities in the area (Suh et al., 2015). The soil fertility is as a result of humus, which is a dark volcanic soil from the uplands/hilly areas that has been washed down from the hills and deposited on river banks or beds of streams (Tematio et al., 2001). Plant material: *Echinops giganteus* CD Adams

Seeds collection, selection and preparation

Seeds were collected from the Western Region of Cameroon, in fields where it grows naturally. Mature fruits were collected from the mother plant growing in the wild, dried for two weeks under natural sunlight then matured seeds with healthy grains were selected for germination. Some seeds were randomly selected for viability test by floatation method. The seeds were placed in a bucket of water at room temperature (Wamegni, 1991; Schaal, 2000). The seeds that sank down were classified as viable seeds, while those that floated were classified as non-viable.

Nursery construction

The field was cleared using a cutlass and ploughed with a hoe. Nursery beds measuring 1 by 4 m were established with distance of 50 cm apart. The entire nursery site was shaded with palm fronds. Seed sowing method was by line broadcast. The Blocks were 1 m apart for each nursery site. Nursery beds were monitored and watered after every one day.

Vegetative propagation of seedling cuttings

The non-mist propagator was partitioned into compartments each was constructed following the design modeled by Leakey et al. (1990). It was a wooden frame enclosed in a single sheet of polythene such that the base was completely water tight. The frame was covered tightly with a single piece polythene and closely fitting lid. The polythene base of the propagator was covered with a thin layer of sand to protect the polythene, and onto this was placed a layer of large stones to a depth of 10-15 cm. This was then covered by successive layers of small stones and gravels to a total depth of 20 cm. The space between stones and gravel was filled with water. The saturated layer of stones and gravel was covered by the rooting medium which consisted of a layer of fine river sand treated with fungicide. The rooting medium was made to remain moist by

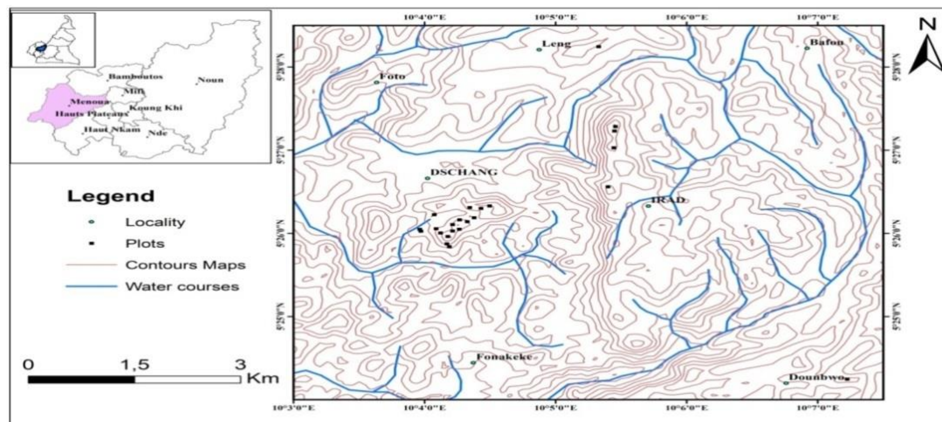

Figure 1. Map of study area in the Menoua Division.

capillarity and was dampened above as necessary. This resulted in a permanently humid environment throughout the propagation period. An open plastic cylinder (tube) of 25 cm long and 4 cm in diameter was vertically inserted in the propagator before filling. This cylinder was used as filling point for the water and allowed a regular check of the water table.

Preparation of cuttings

The plants were allowed to grow for two months at the transplanting sites from which five hundred mature uniformly healthy plants will be used to provide cuttings for this study. This time it was ensured that seedlings attain the necessary vigor. These mature plants were carefully uprooted and cuttings were obtained. Using a sharp knife, cuttings were collected at the stem base [SB], stem middle (SM), stem apex [SA] and root Apex [RA] of 4-6 cm in length each. The cuttings were deepened in to IBA and NAA hormones at different concentration and then put into the sand growth substrate.

For IBA (UN: 2811, LOBAL CHEMIE) 2g of powder hormone was put into 200 ml of alcohol to prepare the mother solution of 10 g/L which was further diluted with alcohol to prepare concentrations (0, 0.5, 1, 2 and 5) g/L; while for NAA (BDH Chemicals Ltd), 0.8 g of powder hormone was put into 80 ml of alcohol to prepare the mother solution of 8 g/L which was further diluted with alcohol to prepare concentrations (0, 0.5, 2 and 4 g/L). The growth hormone concentration range was large reason being that it is the first work done to evaluate the effect of hormone concentration on *E. giganteus* plant

Experimental design

A total of 558 cuttings set in a Complete Randomized Design were used with two Growth Regulator Hormones in the main blocks and four different cutting positions (SB, SM, RA and SA) were tested at the subplot level. At each level, treatments were assigned at random to experimental units so as to have a layout like this; 4 cutting positions *3 repetitions * 2 blocks. Growth hormones were used to enhance rooting; the experiment ran for 16 weeks.

Data collection

For the non-mist propagator experiment, cuttings were assessed for survival, mortality, number of roots, longest root length, shortest root length, and number of leaves.

Data analysis

Data was presented using tables and figures. Data on early growth parameters was subjected to Analysis of Variance (ANOVA) using the statistical programmer XLSTAT where the least significant differences (LSD) between the mean was detected and separated using the Duncan's New Multiple Range Test (DNMRT) at $p \leq 0.05$.

RESULTS

Results on the vegetative propagation of *E. giganteus*

The results gotten for seedling Apex, Meddle and Base were negative since none of them reported (Figure 2), but never the less root apex cuttings had some results as presented.

Effect of NAA concentrations on growth parameters *E. giganteus* root apex

From the results, it was observed that there was no significant difference amongst the growth parameters that were measured, but there was a difference in mortality rate with respect to hormone concentrations. The control experiment that was without hormone gave the best results for NAA hormone which was significantly different from the rest of the other concentrations that had no significant difference amongst themselves (Table 1). For the mortality rate, the lowest mortality rate of 11% was in the control experiment, but when the NAA hormone was used, it was observed a mortality rate of 89% at concentration 1g/L and the rest of the concentrations gave a 100% mortality rate (Figure 3).

Effect of IBA concentrations on growth parameters *E. giganteus* root apex

From our results, we can saw that there was no significant

Figure 2. Stem cuttings sown after being deepened in hormones (a), stem cuttings not regenerated (b).

Table 1. Effect of NAA concentrations on growth parameters *E. giganteus* root head.

Concentrations	NL	NR	SRL	LRL
0 g/L	11.56 ± 5.42[a]	7.11 ±5.82[a]	0.38 ±0.41[a]	1.79 ± 1.62[a]
1 g/L	0.67 ± 1.89[b]	2.22 ± 6.29[b]	0.06 ± 0.16[b]	0.22 ± 0.63[b]
0.5 g/L	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
10 g/L	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
2 g/L	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
5 g/L	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
Pr > F	0.00	0.00	0.00	0.00
SD	yes	yes	yes	yes

*Values indicated by the same letters within the columns are not statistically different at P ≤ 0.05.
NL: Number of leaves, **NR:** Number of roots, **SRL:** Shortest root length, **LRL:** Longest root length

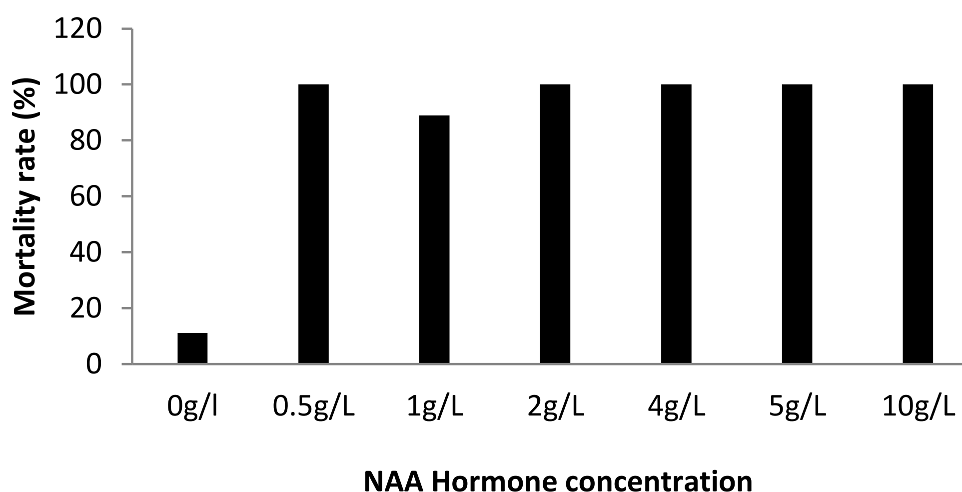

Figure 3. Effect of NAA concentrations on mortality rate of *E. giganteus* root head.

difference amongst the growth parameters that were measured, likewise in hormone concentrations. The control experiment that was without hormone gave us the highest results for IBA hormone which was not significantly different from the rest of the other concentrations that had no significant difference amongst themselves too (Table 2). For the mortality rate, the lowest mortality rate of 33% was in the control

Table 2. Effect of IBA concentrations on growth parameters *E. giganteus* root head.

Concentrations	NL	NR	SRL	LRL
0 g/L	5.89 ± 4.70[a]	2.89 ± 3.25 [a]	0.42 ± 0.86[a]	1.64 ± 2.13[a]
8 g/L	2.67 ± 4.99[ab]	2.00 ± 4.00 [a]	0.06 ± 0.16[a]	0.34 ± 0.97[b]
0.5 g/L	1.67 ± 2.49[b]	1.22 ± 2.39[a]	0.22 ± 2.39[a]	0.24 ± 0.69[b]
4 g/L	2.00 ±3.77[ab]	1.44 ± 3.17[a]	0.03 ±0.09 [a]	0.22 ±0.63 [b]
2 g/L	1.00 ± 2.00 [b]	0.89 ± 1.73[a]	0.16 ±0.44 [a]	0.23 ± 0.66[b]
Pr > F	0.11	0.71	0.58	0.08
SD	No	No	No	No

*Values indicated by the same letters within the columns are not statistically different at P ≤ 0.05.

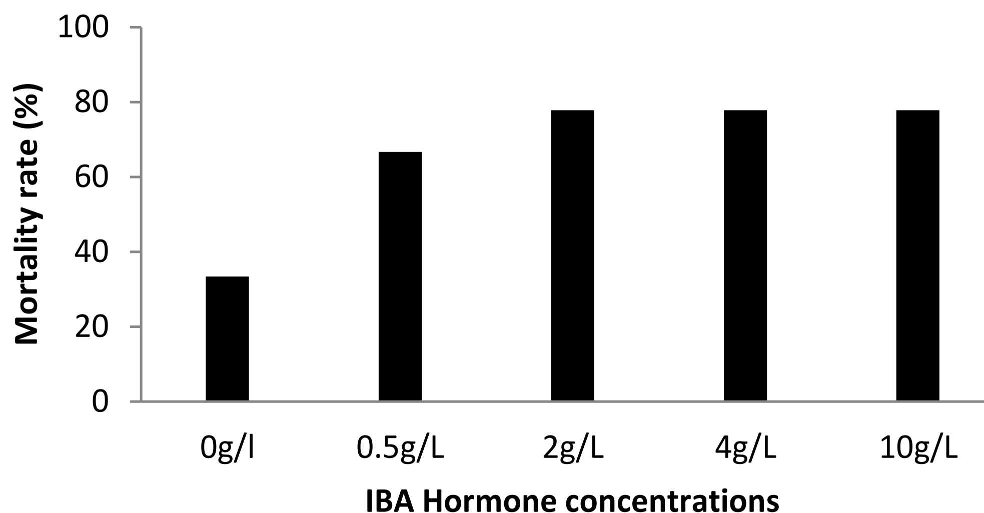

Figure 4. Effect of IBA concentrations on the mortality rate *E. giganteus* root head.

experiment, but when the IBA hormone was used, we observed a mortality rate of 67% at concentration 0.5g/L and the rest of the concentrations gave a 78% Mortality rate each (Figure 4).

Comparism between experiments in and out of the propagator

Table 3 shows that we had significant differences for growth parameters; the number of leaves and longest root length for both experiments and we also saw that when the experiment was carried out of the propagator and without (WOUT) the use of any plant growth hormone, the results were best (Figure 5). This is clearly explained by their mortality rates. For the mortality rate, the lowest mortality rate of 0% was in the experiment out of the propagator, followed by NAA control experiment in the propagator with a mortality rate of 25%. Also, root development was better for root apex cuttings out of the propagator than in the propagator (Figure 6).

DISCUSSION

Target 8 of the Global Strategy for Plant Conservation has a stated goal of ‘‘At least 75% of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20% available for recovery and restoration programs of them included in recovery and restoration programs’’ (Sharrock, 2012). As Pence (2011) noted, Target 8 encompasses the following two goals of ex situ conservation: propagation of plants for recovery of wild populations and to provide backup collections of plants for restorations should wild plants be lost. Seed-based methods are the most efficient for ex situ preservation provided there is enough seed available and germination procedures are adequate (Pence, 2011). Additionally, preservation can be accomplished using propagation by cuttings or other vegetative methods.

In the experiments, hormone concentrations inhibited rooting and regeneration of buds but increased mortality in all cutting positions (Apex, Middle, Base and Root Apex) of *E. giganteus*. The differences in root growth may

Table 3. Comparism between experiments in and out of the propagator.

Parameter	NL	NR	SRL	LRL
WOUT	13.28±7.75 [a]	5.06 ±5.40[a]	1.59 ±2.05[a]	5.73 ±4.44[a]
NAA	11.56± 5.42[b]	3.33 ±3.19[a]	0.39 ±0.41[a]	1.79 ±1.62[b]
IBA	5.89±4.70 [b]	1.67±1.83[a]	0.42 ±0.86[a]	1.64 ±2.13 [b]
Pr > F	0.04	0.18	0.09	0.01
SD	yes	No	No	yes

*Values indicated by the same letters within the columns are not statistically different at P ≤ 0.05.

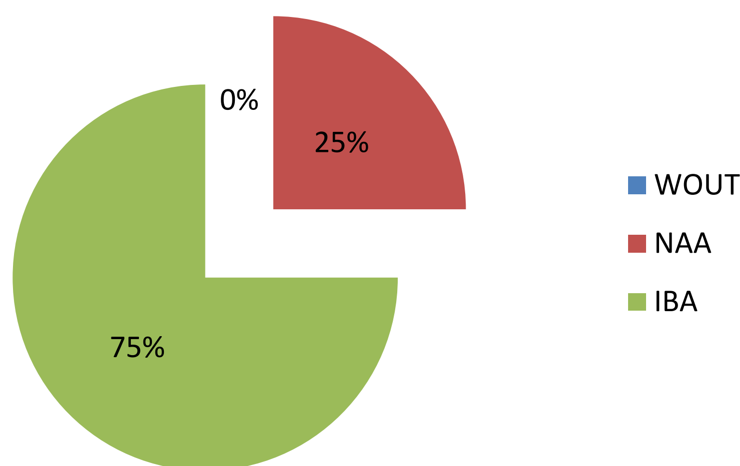

Figure 5. Comparism of mortality rate between experiments in and out of the propagator.

Figure 6. Root head results out of the propagator (a) and in the propagator (b).

be due to the differential effects of the growth regulators on metabolites translocation and carbohydrates metabolism (Abubakar et al., 2019). This was contrary to the results of Monteiro et al. (2010) in evaluation of sweet potato cuttings in sub soil with five concentrations of IBA, Kanmegne et al. (2015) on *Cola anomala* cuttings who reported that apical cuttings result in lower mortality than basal cuttings and those reported on the vegetative propagation by cuttings of *Lovoa trichilioides* (Tchoundjeu and Leakey, 2001) which show that the mortality rate increases from the apical nodes to the basal nodes.

However, our results for vegetative propagation of *E.*

giganteus are in line with those reported on *Khaya ivorensis* cuttings (Tchoundjeu and Leakey, 1996) which suggest that the mortality rate of apical cuttings is higher than that of basal cuttings. These different results indicate that there are interspecific variations in the response of cuttings to different positions on the mother stem, but the determinism of these variations is not well known (Kanmegne et al., 2015). High concentrations of IBA would therefore have a toxic effect on the survival of *E. giganteus* cuttings. This hypothesis is supported by the work of Ezzili and Bajouai (2000) and Houar et al. (2014) on the effect of different types of auxins (IAA, IBA, and NAA) on the rooting of *Simmondsia chinensis* cuttings. This work reveals that the survival of cuttings is inversely proportional to the rate of applied auxins.

In their work, Tchoundjeu and Leakey (2001) have shown that in *Lovoa trichilioides* cuttings from apical knots take root much better than cuttings from basal knots of the same stem. In *E. giganteus*, the rooting rate did not increases with the concentration of IBA, not sharing the same idea with researchers who said IBA has a positive effect on the induction of adventitious root formation (Leakey, 2004; Kanmegne et al., 2017). The results are in line with those obtained by Mapongmetsem et al. (2012) where IBA did not improve the percentage of rooting of cuttings in *Vitex doniana*. These contradictory results indicate that the need for exogenous auxin supply for rooting induction varies from one species to another. This variation may be partly due to differences in the concentrations of endogenous auxin in different plants at the time of excision of cuttings (Leakey, 2004; 2014).

Conclusion

Vegetative propagation is possible for *E. giganteus* without the use of any hormone. This method appears to be promising for establishing cultivated stands, because it permits the increase of improved lines. Traits such as resin content and composition, and regrowth after harvest are some of the most important characteristics to select for. The vegetative propagation process through cuttings of *E. giganteus* is most recommended out of the propagator in an open field during the raining season, without any growth hormone or fertilizers. This results to a great percentage of survived cuttings, rooted cuttings, cuttings with buds, producing a good number of leaves and a healthy plant. Inadequacies of quality planting materials remain the major constraints for establishing large scale plantation programs of *E. giganteus* in Cameroon and other tropical countries. Due to scarcity of seeds, vegetative propagation through root head cutting can be a better and helpful option of multiplication. Considering farmers perception regarding the regeneration and domestication of *E. giganteus,* quality planting materials of *E. giganteus* will inspire farmers to plant more *E. giganteus* at their homesteads as well as to establish nurseries for commercial production.

Recommendation

The Ministry of Environmental Protection should provide uniform training to those involved in the regeneration of *E. giganteus* with a clearly defined organizational framework within which there should be more collaboration among the bodies involved (those from the ministry and the worker) to ensure easy accessibility to resources for effective work done, information flow, supervision and monitoring.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

The costunolide biosynthesis enzymes of *Artemisia glabella* Kar. et Kir.: Determination of the nucleotide sequences of the mRNA

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***Artemisia glabella* Kar. et Kir. is a source of sesquiterpene lactone arglabin, which has antitumor, radiosensitizing and immunomodulatory activity. Studying the biosynthesis of arglabin and its derivatives will allow us to develop the biotechnological basis for its production, thereby increasing its availability. The precursor to most sesquiterpene lactones is costunolide. The purpose of these studies was to detect and determine the nucleotide sequences of mRNA enzymes germacrene A synthase (GAS, EC 4.2.3.23), germacrene A oxidase (GAO, EC 1.14.14.95) and costunolide synthase (COS, EC 1.14.13.120), involved in the biosynthesis of costunolide in *A. glabella*. As a result of studies, mRNA was isolated from various forms (intact plant, regenerant plant, callus) of *A. glabella*. Using specific primers, mRNA fragments of genes encoding sesquiterpene lactones biosynthesis enzymes were amplified and their nucleotide sequences were determined. A comparative analysis of the obtained sequences showed their high (>90%) identity with the genes of GAS, GAO and COS of other representatives of the family Asteraceae. It was revealed that enzymes are expressed both in an intact plant and in calluses and regenerant plants obtained *in vitro*.**

Key words: *Artemisia glabella* Kar. et Kir., mRNA, cDNA, sequencing, germacrene A synthase, germacrene A oxidase, costunolide synthase.

INTRODUCTION

Sesquiterpenoids of the guaiane series belong to fairly widespread substances of the plant kingdom. A particularly large number of guaiane compounds are included in the group of sesquiterpene lactones called guaianolides. α-Methylene guaianolides have a bitter taste, are toxic, and exhibit cytotoxic and antitumor properties. Their molecules, as a rule, are polyfunctional and many of them are characterized by the presence of exocyclic unsaturation (Fischer et al., 1979; Frederick, 1982).

Sesquiterpene lactones are derivatives of isopentenyl diphosphate (IPP), which can be synthesized in two

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different ways: the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. In the MEP pathway, glyceraldehyde-3-phosphate and pyruvic acid are converted to IPP and DMAPP by seven enzymes. In the MVA pathway, acetyl-CoA is converted to IPP through six steps followed by IPP isomerase (IPPI). Two IPPs and one DMAPP are then converted to farnesyl diphosphate (1) (FPP), the precursor of all sesquiterpenes, by farnesyl diphosphate synthase (FPS). FPP (1) can be converted to sesquiterpenes using sesquiterpene synthases FPP (1).

Sesquiterpene lactones are formed in the plant organism by oxidation of the methyl part of the isopropyl group attached to the main carbon skeleton. Cyclization of farnesyldiphosphate (1) gives (+)- germacrene A (2). Oxidation of the isopropenyl side chain by (+)-germacrene A-hydroxylase to primary alcohol and further oxidation by NAD (P)+- dependent dehydrogenases gives germacrene acid (3). This is followed by hydroxylation at the C-6 position and subsequent lactonization leads to (+)- costunolide (4). It is assumed that the second stage of the cyclization of germacranolides to the guaianolide skeleton proceeds through epoxidation or hydroxylation of the costunolide skeleton (Ma et al., 2019).

As a result of research on the homology of nucleotide sequences with known sesquiterpene monooxygenases, six promising cytochrome P450 contigs (actin, GAS, GAO, COS, parthenolide synthase and costunolide 3β-hydroxylase) were identified and selected for functional characterization. A new cytochrome P450, cauniolide synthase, which catalyzes the formation of guaianolide cauniolide (5) from the germacranolide substrate costunolide (4), has been characterized. Unlike most cytochromes P450s, cauniolide synthase has a unique mechanism of action, combining stereoselective hydroxylation of costunolide (4) at the C-3 position with elimination, cyclization, and regioselective deprotonation (Liu et al., 2018).

One of the promising guaianolides is arglabin (6), isolated for the first time from *Artemisia glabella* Kar. et Kir., an endemic species of wormwood (*Artemisia* L.), which grows exclusively in Central Kazakhstan. *A. glabella* is a source of a number of biologically active compounds such as sesquiterpene lactones and essential oils (Adekenov et al., 1982; Adekenov et al., 1995). Arglabin (6) is the active substance of the anticancer drug “Arglabin”, which was developed at the International Research and Production Holding “Phytochemistry” and is produced on an industrial scale by the Karaganda Pharmaceutical Plant (Karaganda, Republic of Kazakhstan) (Adekenov, 2001, 2015).

Arglabin (6) is found in all organs of *A. glabella* and throughout the growing season (Adekenov et al., 1995). Its quantitative accumulation is observed during the budding period in leaves (1.90%) and buds (1.56%) (Mantler et al., 2020). However, the biosynthesis stages of the arglabin molecule in a plant organism at the genetic level are studied insufficiently.

Promising approach to obtain arglabin (6) can be the reconstruction of the biosynthetic pathway in a heterologous system. However, despite the widespread medical use of arglabin (6), the biosynthetic pathway in plants remains unexplored. Adekenov and Bouwmeester (2015) described a possible pathway for arglabin biosynthesis in *A. glabella*: farnesyldiphosphate (1), germacrene A (2), germacrene A-ol (7), germacrene A-on (8), germacrene acid A (3), costunolide (4), cauniolide (5), and arglabin (6) (Figure 1).

The formation of germacrene A (2) as a result of the cyclization of farnesyl diphosphate (1) catalyzed by germacrene A synthase (GAS) is a generally recognized step in the biosynthesis of sesquiterpenes (Xu and Dickschat, 2020). GAS was isolated and characterized from a number of plants of the family Asteraceae (Bouwmeester et al., 2002; Majdi et al., 2011; Menin et al., 2012; Pazouki et al., 2015; Nguyen et al., 2016). The next step in biosynthesis is the sequential oxidation of germacrene A (2) by germacrene A oxidase (GAO) and costunolide synthase (COS). As a result, germacrene acid (3) and costunolide (4) are formed, respectively (Nguyen et al., 2019; de Kraker et al., 2002; Ikezawa et al., 2011).

In previous studies, a number of genes encoding sesquiterpene enzymes that control key steps in secondary metabolic pathways have been characterized from a number of herbal plant species. However, the genes responsible for enzymatic processes in guaianolide biosynthesis have not been identified. Therefore, the isolation and characterization of genes involved in the biosynthesis of arglabin (6) in *A. glabella* is a promising direction of research for the reconstruction of the process in a heterologous system. The main objectives of this study were the isolation of genes encoding the GAS, GAO and COS in *A.glabella* and the determination of the nucleotide sequences of mRNA, as well as the assessment of the expression of these genes at the level of transcription in regenerated plants and callus tissues.

MATERIALS AND METHODS

Plant

The intact plant of *A. glabella* Kar. et Kir. in the budding phase under *ex situ* conditions, the regenerant plant and callus tissues obtained *in vitro* culture in the biotechnology laboratory of JSC “International Research and Production Holding “Phytochemistry” were used. Leaves and flower buds were collected and immediately frozen in liquid nitrogen and stored at -80°C (three biological replicates for each tissue were used).

RNA isolation: cDNA synthesis

For total RNA extraction, fresh or freezed plant material (about 100 mg) immediately transferred to 200 µl Tryzol reagent in 1.5 ml

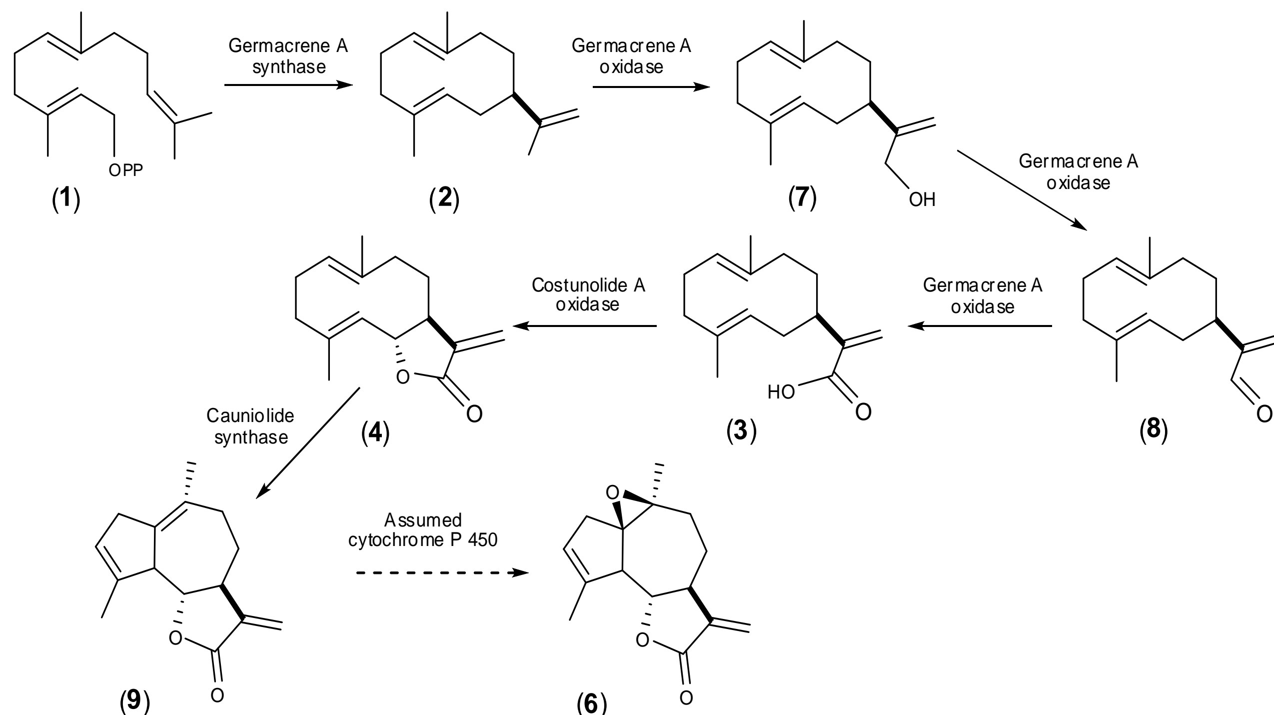


Figure 1. Estimated pathway of arglabin biosynthesis.

microcentrifuge tube. Cells were disrupted mechanically with a pestle in the presence of carborundum. After cell disruption, an additional 800 µl Tryzol reagent was added. All subsequent steps were carried out as described in the manual.

mRNA was isolated from total RNA using the Dynabeads® mRNA DIRECT™ Purification Kit (Invitrogen, USA) according to the manufacturer's protocol.

cDNA synthesis was performed using the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific, USA), according to the manufacturer's protocol, with oligo (dT) primer.

Amplification and sequencing of fragments of targeted genes

Amplification of cDNA fragments of targeted genes was carried out using specific primers designed as part of these studies. A 50 µl reaction mixture contained: 5 µl 10×PCR buffer (Silex, Russia), 1 µl 10 mM dNTPs (NEB), 0.1 µl cDNA (100 ng/µl), 1 µl of each primer (20 pM/µl), 0.25 µl Taq DNA polymerase (1.25 units, Sileks). Amplification was carried out under the following conditions: at 94°C 5 min; then 30 cycles at 94°C 1 min; at 50°C 1 min; at 68°C 2 min; final elongation at 68°C 7 min.

PCR products were cloned in pJet1.2 vector using the Clone JET PCR Cloning Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Competent cells of *Escherichia coli* TOP10 were transformed with ligation mix. Presence and size of insertions in plasmid vector was determined by PCR analysis of *E. coli* TOP10 colonies according to the manufacturer's instructions for CloneJET PCR Cloning Kit (Thermo Scientific). Plasmid DNA was isolated from *E. coli* TOP10 using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol.

Nucleotide sequences of targeted genes were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX), on the DNA sequencer Genetic Analyser 3130xl, Applied Biosystems, Hitachi, Japan. Nucleotide and their relevant amino acid sequences were analyzed with the help of the software Vector NTI 10.0.1, Invitrogen. The GAS/GAO/COS nucleotides from the GenBank databases were used for comparison.

Phylogenetic analyses

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

RESULTS AND DISCUSSION

Design of primers

The design of primers for amplification of target genes was carried out based on the analysis of the nucleotide and amino acid sequences of GAS, GAO and COS in members of the Asteraceae family presented in the GenBank of The National Center for Biotechnology Information, U.S. National Library of Medicine.

Amino acid sequences of GAS, GAO, COS are presented in GenBank for 17, 11 and 8 species of the Asteraceae family, respectively. Two forms of GAS were found: short ~ 560 amino acids (a.a.) and long ~ 580 a.a. The average the length of the amino acid sequences of

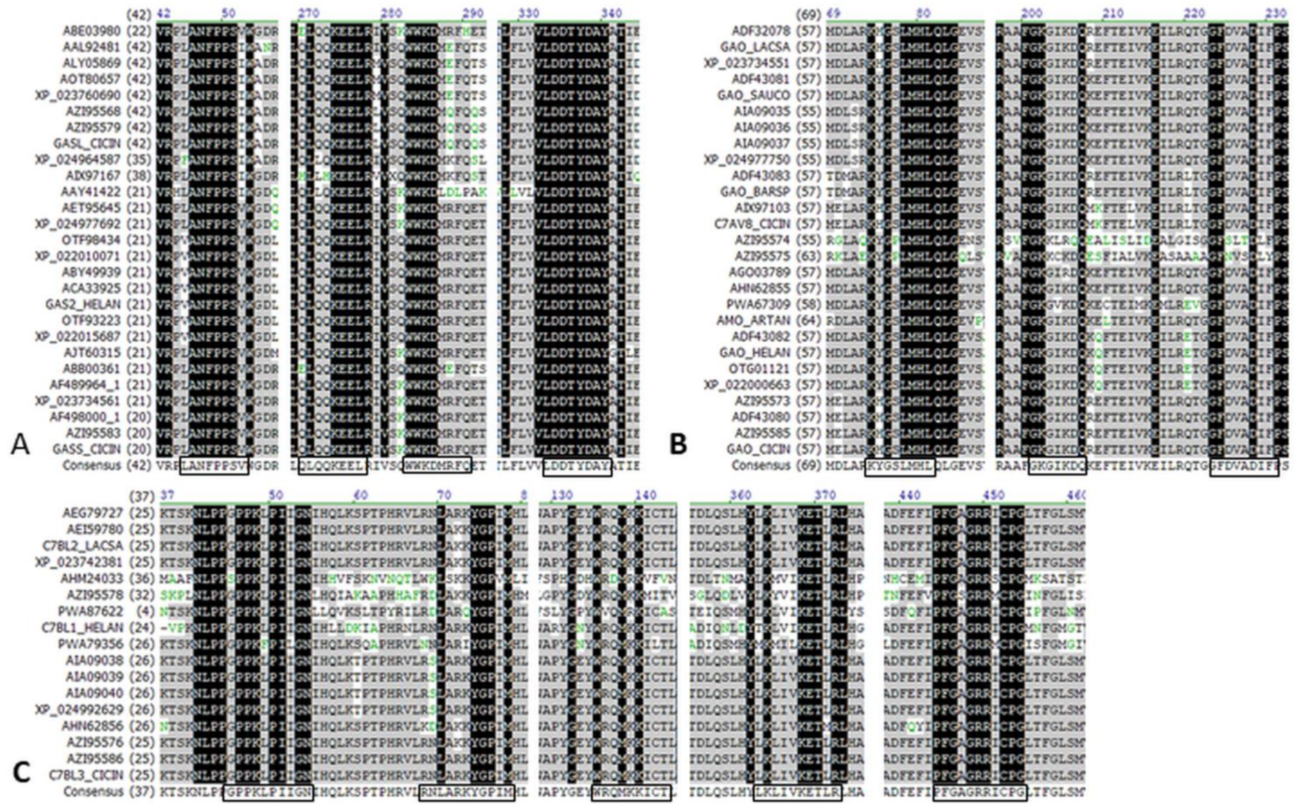


Figure 2. Alignment of amino acid sequences of GAS (A), GAO (B), and COS (C) (conservative sites highlighted).

Table 1. Primers used in this study.

Name	Target peptide	Primer sequence 5'-3'	PCR-product, bp
GAS-F1	LQLQQKEEL	tggagctacgacaaaaagaagaactg	216-885
GAS-F2	LANFPPSVW	ctggccaactttcctccttcagtatgg	
GAS-R1	LDDTYDAY	ataagcatcatatgtgtcatcta	
GAS-R2	WWKDMRF	aaacctcatgtccttccacc	
GAO-F1	KYGSLM	gccagaaagtatggatctttaatgca	405-469
GAO-R1	GKGVKDQ	ctggtccttgactccttttcc	
GAO-R2	GFDVADIF	gaaagatatctgccacatcaaagcc	
COS-F1	GPPKLPIIGN	gggccgccaaaactacccataatcggaaac	300-1200
COS-F2	LKLIVKETLR	ctgaaattaatagtaaaagaaactctgagg	
COS-F3	RNLAKKYGPIM	gaaacttagccaagaaatatggccccatcatg	
COS-R1	PFGAGRRICPG	acaaattctccggccggcaccgaacgg	
COS-R2	WRQMKKICTLE	ctccaaggtgcaaatcttcttcatctgcctcca	

GAO is 488 a.a. and COS is 494 a.a.

Conserved short peptides were identified based on the multiple alignments of the amino acid sequences of GAS, GAO, COS (Figure 2). These peptides became targets for the design of specific primers (Table 1).

Amplification and sequencing of target genes

Fragments of target genes of *A. glabella* (Ag) were amplified using specific primers (Figure 3A) and sequenced. The obtained sequences of mRNA fragments

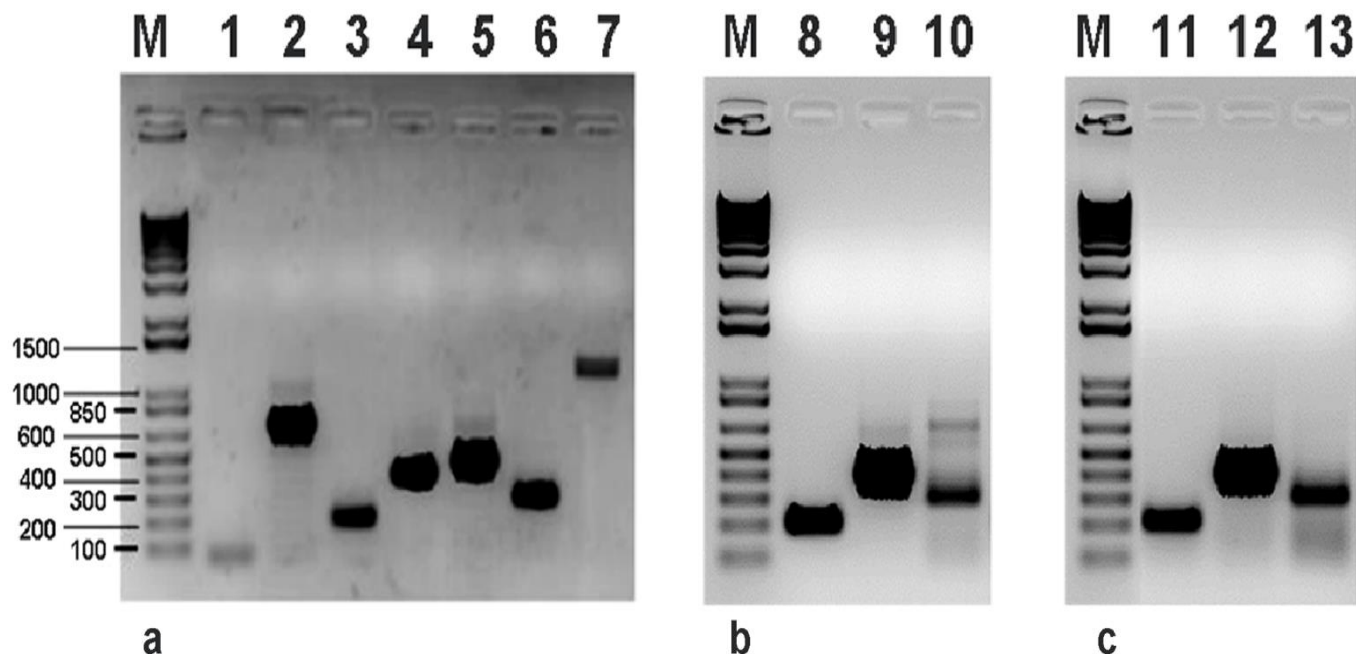


Figure 3. Results of target genes fragments amplification using cDNA obtained from various tissues of *A. glabella* Kar. et Kir. as a template. a - cDNA of an intact plant, b - cDNA of a regeneranted plant, c - cDNA of callus tissues; M - marker of the length of DNA fragments (1 Kb Plus DNA Ladder, Invitrogen); 1 - primers GAS-F2/GAS-R1 (885 bp); 2 - primers GAS-F1/GAS-R2 (216 bp); 3,8,11 - primers GAS-F2/GAS-R2 (737 bp), 4,9,12 - primers GAO-F1/GAO-R1 (405 bp); 5 - primers GAO-F1/GAO-R2 (469 bp); 6 - primers COS-F1/COS-R1 (1200 bp); 7,13 - primers COS-F3/COS-R2 (300 bp).

of AgGAS, AgGAO, and AgCOS were deposited in GenBank with IDs MT276314, MT276315, and MT276313, respectively.

Phylogenetic analysis

Pairwise protein sequences comparison of putative AgGAS, AgGAO and AgCOS with all known enzymes shows wide range of homology.

In silico analysis revealed that putative AgGAS has about 40 to 90% homology at protein level with annotated germacrene A synthases. It has highest identity (90%) with *Tanacetum parthenium* germacrene synthase (TpGAS) and lowest identity (40%) with *Solidago canadensis* germacrene synthases (ScGAS) (Figure 4A). Neighbour joining algorithm generated phylogenetic tree has combined AgGAS, AaGAS and TpGAS into one evolutionary lineage (Figure 4B). *Tanacetum* and *Artemisia* are members of the taxonomic tribe Anthemideae.

Phylogenetic analysis of AgGAO and AgCOS showed high homology with the corresponding enzymes of *T. parthenium*, 97 and 95%, respectively (Figures 5A and 6A). Neighbour joining algorithm generated phylogenetic tree showed separate evolutionary lineage of AgGAO and AaGAO (Figure 5B). However, AgCOS was combined in one lineage with TpCOS (Figure 6B).

Expression of target genes in plant tissues

Expression of AgGAS, AgGAO and AgCOS was evaluated at the transcription stage in regenerant plant and callus tissues obtained *in vitro.* As a result, we found that mRNA of costunolide (4) biosynthesis enzymes is present both in regenerant plants and in callus tissues (Figure 3B and C). Regenerated plants and callus tissue can also provide an alternative source for arglabin (6) production.

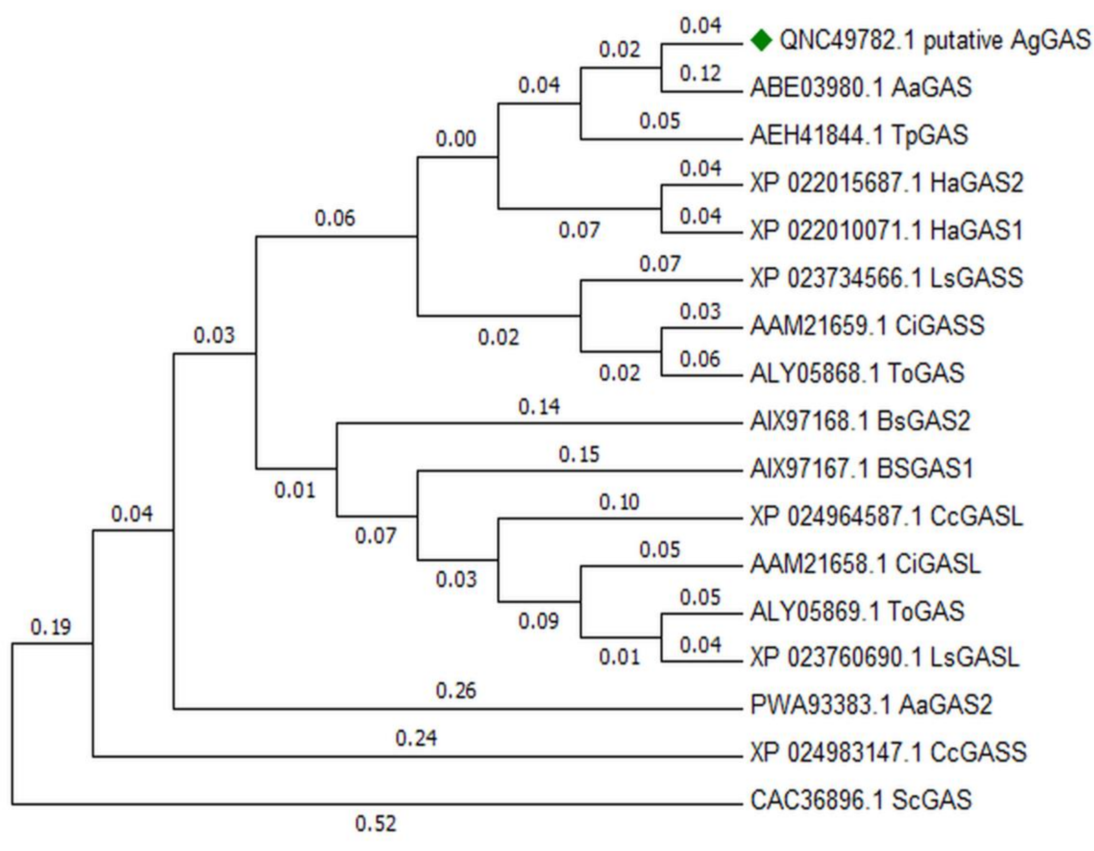
Conclusion

Thus, for the first time the nucleotide sequences of mRNA fragments encoding AgGAS, AgGAO and AgCOS were determined. A comparative analysis of the obtained sequences showed their high (> 90%) identity with the annotated GAS, GAO and COS of other members of the family Asteraceae. It was found that AgGAS, AgGAO and AgCOS are expressed both in the intact plant, as well as in calluses and regenerated plants obtained *in vitro*.

The results obtained provide a basis for further study of the biosynthetic pathway of arglabin (6) in plants. Isolation and characterization of enzymes will make it possible to reconstruct the biosynthesis of arglabin (6) in a heterologous system and to simplify the methods of its

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
QNC49782.1_putative_AgGAS	1		87	70	68	86	84	67	84	69	62	68	84	83	90	40	77	57
ABE03980.1_AaGAS	2	0.15		65	64	79	77	63	79	64	60	61	76	76	83	35	71	51
AIX97167.1_BSGAS1	3	0.40	0.49		80	73	72	79	73	80	63	78	73	72	71	39	76	51
AAM21658.1_CiGASL	4	0.42	0.48	0.34		72	72	93	71	94	62	79	71	71	70	38	74	47
AAM21659.1_CiGASS	5	0.18	0.25	0.35	0.37		94	71	90	72	67	69	88	89	87	38	73	56
ALY05868.1_ToGAS	6	0.20	0.28	0.39	0.38	0.08		71	88	72	66	69	86	87	85	37	80	54
ALY05869.1_ToGAS	7	0.44	0.50	0.35	0.11	0.39	0.40		70	92	61	79	70	70	68	38	73	48
XP_023734566.1_LsGASS	8	0.19	0.26	0.40	0.42	0.12	0.15	0.42		71	66	69	85	86	86	38	79	55
XP_023760690.1_LsGASL	9	0.42	0.48	0.32	0.10	0.38	0.39	0.10	0.41		62	79	71	71	70	38	74	48
XP_024983147.1_CcGASS	10	0.49	0.58	0.50	0.55	0.43	0.45	0.55	0.43	0.54		60	66	66	66	37	68	50
XP_024964587.1_CcGASL	11	0.38	0.47	0.26	0.24	0.32	0.32	0.24	0.37	0.25	0.53		69	68	67	38	72	50
XP_022015687.1_HaGAS2	12	0.20	0.30	0.38	0.40	0.18	0.21	0.42	0.22	0.40	0.46	0.35		95	86	38	78	53
XP_022010071.1_HaGAS1	13	0.22	0.30	0.40	0.41	0.17	0.19	0.43	0.20	0.42	0.48	0.37	0.08		86	38	78	53
AEH41844.1_TpGAS	14	0.11	0.18	0.42	0.41	0.16	0.19	0.44	0.18	0.42	0.46	0.38	0.19	0.19		37	78	55
CAC36896.1_ScGAS	15	0.94	1.02	0.93	1.02	0.88	0.93	0.98	0.93	0.98	0.94	0.98	0.91	0.94	0.98		38	32
AIX97168.1_BsGAS2	16	0.30	0.40	0.35	0.40	0.28	0.30	0.41	0.29	0.39	0.46	0.32	0.33	0.34	0.31	0.93		56
PWA93383.1_AaGAS2	17	0.42	0.53	0.54	0.58	0.42	0.46	0.58	0.45	0.58	0.53	0.53	0.48	0.46	0.44	1.01	0.42	

A



B

Figure 4. Pairwise protein sequence comparison (A) and phylogenetic tree (B) of putative *Artemisia glabella* germacrene A synthase (◆ AgGAS) and annotated germacrene A synthases. AaGAS; AaGAS2: *Artemisia annua*, TpGAS: *Tanacetum parthenium*, HaGAS1; HaGAS2: *Helianthus annuus*, LsGASS; LsGASL: *Lactuca sativa*, CiGASS; CiGASL: *Cichorium intybus*, ToGAS: *Taraxacum officinale*, BsGAS1; BsGAS2: *Barnadesia spinose*, CcGASS; CcGASL: *Cynara cardunculus var. scolymus*, ScGAS: *Solidago canadensis*. A: Lower left triangle shows pairwise evolutionary distance and upper right triangle shows percentage identity.

		1	2	3	4	5	6	7	8	9	10	11	12
AIX97103.1_LsGAO2	1		83	84	83	83	84	83	83	74	82	87	85
AGO03789.1_TcGAO	2	0.14		100	89	90	90	88	89	90	89	84	96
AHN62855.1_TpGAO	3	0.13	0.01		89	90	90	89	89	91	89	84	97
AIA09036.1_CcaGAO	4	0.15	0.05	0.05		89	99	89	89	82	87	83	92
XP_023734551.1_LsGAO	5	0.11	0.08	0.07	0.08		90	98	98	83	89	84	90
XP_024977750.1_CcsGAO	6	0.14	0.04	0.03	0.01	0.07		90	90	83	87	84	94
AZI95573.1_CeGAO	7	0.12	0.09	0.08	0.08	0.01	0.07		99	81	89	84	89
AZI95585.1_CiGAO	8	0.12	0.09	0.08	0.08	0.01	0.07	0.00		81	89	84	89
PWA67309.1_AaGAO	9	0.27	0.14	0.14	0.19	0.21	0.17	0.22	0.22		83	75	87
ADF43082.1_HaGAO	10	0.15	0.07	0.07	0.09	0.10	0.08	0.11	0.11	0.18		81	92
ADF43083.1_BsGAO	11	0.11	0.09	0.09	0.11	0.09	0.09	0.09	0.09	0.22	0.14		89
QNC49783.1_putative_AgGAO	12	0.16	0.04	0.03	0.08	0.11	0.07	0.11	0.11	0.14	0.09	0.11	

A

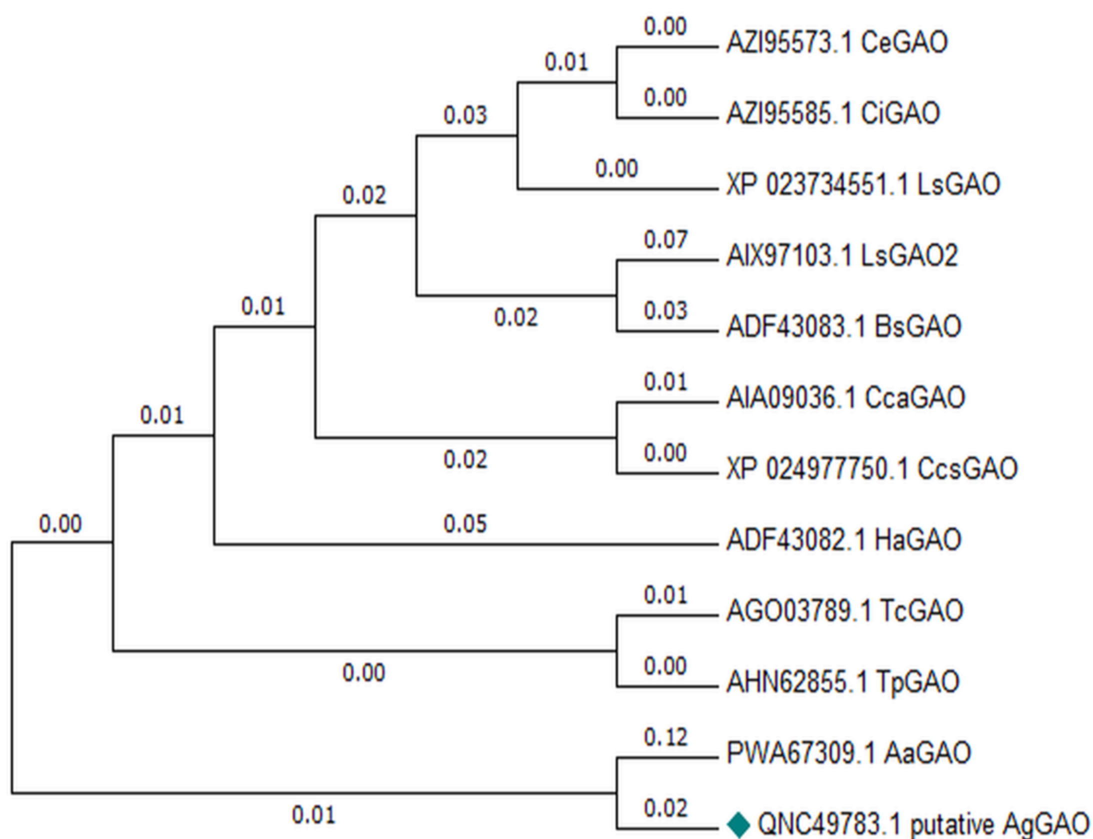


Figure 5. Pairwise protein sequence comparison (A) and phylogenetic tree (B) of putative *Artemisia glabella* germacrene A oxidase (◆ AgGAO) and annotated germacrene A oxidases. CeGAO: *Cichorium endivia*, CiGAO: *Cichorium intybus*, LsGAO, LsGAO2: *Lactuca sativa*, BsGAO: *Barnadesia spinose,* CcaGAO: *Cynara cardunculus var. altilis*, CcsGAO: *Cynara cardunculus var. scolymus*, HaGAO: *Helianthus annuus*, TcGAO: *Tanacetum cinerariifolium*, TpGAO: *Tanacetum parthenium*, AaGAO: *Artemisia annua*. A: Lower left triangle shows pairwise evolutionary distance and upper right triangle shows percentage identity.

		1	2	3	4	5	6	7	8	9
AEI59780.1_LsCOS	1		67	44	96	97	89	89	88	89
PWA79356.1_AaCOS	2	0.38		39	67	67	67	67	65	67
XP_021984083.1_HaCOS	3	0.81	0.91		43	43	43	43	42	45
AZI95586.1_CiCOS	4	0.02	0.37	0.80		100	88	88	88	89
AZI95576.1_CeCOS	5	0.02	0.37	0.80	0.00		88	88	88	89
AIA09040.1_CcsCOS	6	0.11	0.38	0.80	0.11	0.11		100	86	89
AIA09039.1_CcaCOS	7	0.11	0.38	0.80	0.11	0.11	0.00		86	89
AHN62856.1_TpCOS	8	0.11	0.39	0.79	0.11	0.11	0.12	0.12		95
QNC49781.1_putative_AgCOS	9	0.12	0.40	0.80	0.12	0.11	0.12	0.12	0.05	

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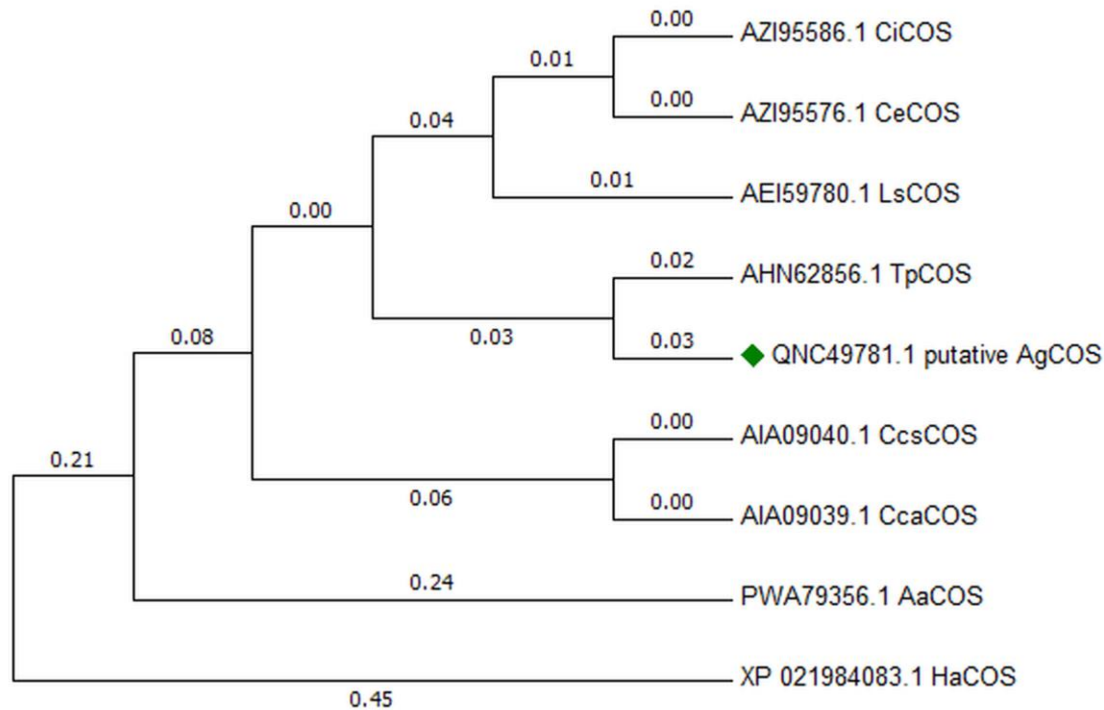

Figure 6. Pairwise protein sequence comparison (A) and phylogenetic tree (B) of putative *Artemisia glabella* costunolide synthase (◆AgCOS) and annotated costunolide synthases. CiCOS: *Cichorium intybus*, CeCOS: *Cichorium endivia*, LsCOS: *Lactuca sativa*, TpCOS: *Tanacetum parthenium*, CcsCOS: *Cynara cardunculus var. scolymus*, CcaCOS: *Cynara cardunculus var. altilis*, AaCOS: *Artemisia annua,* HaCOS: *Helianthus annuus*. A: Lower left triangle shows pairwise evolutionary distance and upper right triangle shows percentage identity.

preparation for pharmaceutical production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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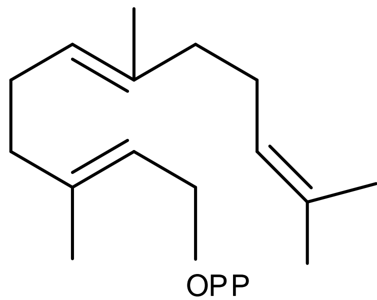
SUPPLEMENTARY



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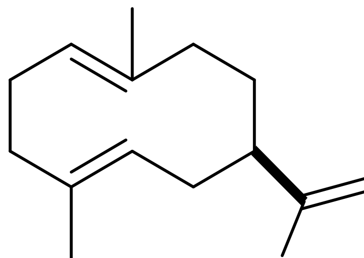

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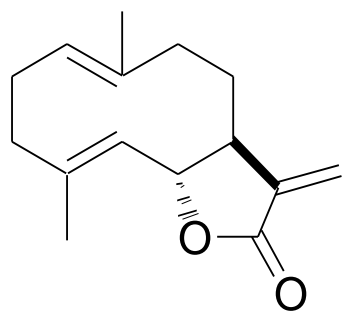

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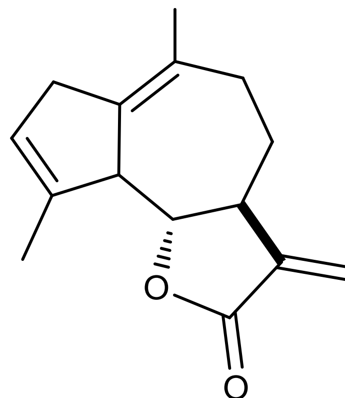

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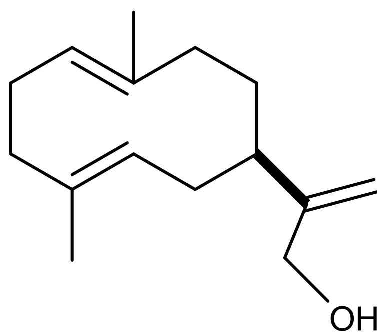

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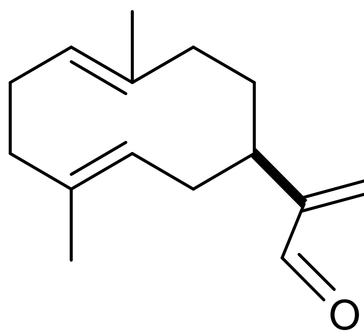

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Full Length Research Paper

Salinity resistance strategy of okra (*Abelmoschus esculentus* L. Moench) cultivars produced in Benin Republic

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Salinity negatively influences the physiology and productivity of plants which develop different strategies to resist to this stress. This study aims to evaluate the implication of sodium (Na), potassium (K), proline and soluble sugars accumulation in salt resistance of okra local cultivars after two weeks exposure to 0, 30, 60, 90 and 120 mM NaCl concentrations. Results revealed that the aerial part growth reduction under salt stress was more accentuated in the salt sensitive cultivar *Keleya* than the salt resistant *Yodana*. Na^+ accumulation in leaves was more accentuated in *Keleya* than *Yodana* whereas proline accumulation was more accentuated in both leaves and roots of *Yodana* than *Keleya*. K^+ content decrease was more accentuated both in leaves and roots of *Keleya* than *Yodana*. Consequently, the decrease in ionic selectivity ratio (K/Na) was more accentuated in the salt sensitive cultivar *Keleya* than the salt resistant *Yodana* in both leaves and roots. Soluble sugars accumulation in leaves depends on the NaCl concentration. Results indicated that the relative salinity resistance of cultivar *Yodana* is associated with sodium ions exclusion from leaves, the maintaining of good accumulation of potassium ions and a good K^+/Na^+ selectivity ratio, and the accumulation of high amounts of proline.

Key words: *Abelmoschus* species, NaCl, sodium, potassium, soluble sugars, proline.

INTRODUCTION

Salt stress is one of the major environmental constraints limiting agricultural productivity (Wei et al., 2003). Salinity is the buildup of soluble salts by which saline soils are formed (Levy and Syvertsen, 2004). It was established in several studies that plant growth is compromised by salinity at all stages of development, but sensitivity varies greatly at different stages (Akram et al., 2002; Akinci et al., 2004; Gandonou and Senhaji, 2015; Loko et al., 2020).

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Gama et al. (2007) reported that plants growing under saline conditions are affected in three ways: reduced water potential in root zone causing water deficit, phytotoxicity of ions such as Na^+ and Cl^- and nutrient imbalance (Ashraf and Foolad, 2007) depressing uptake and transport of nutrients. In saline environment, plant growth is affected by complex interaction of hormones, osmotic effects, specific ion effects and nutritional imbalances, probably all occur simultaneously (Arbona et al., 2005). Although most, if not all crop plants are glycophyte species, their overall responses to increased natrium chloride (NaCl) dose appear to be species specific (Levitt, 1980; Lutts et al., 1995; Karikalan et al., 1999; Lakra et al., 2006; Chukwu and Okpe, 2006; Gandonou et al., 2012). In addition, within the same given species, a substantial variation in salt sensitivity can appear in cultivars (Gandonou et al., 2012; Abbas et al., 2014; Sounou et al., 2021). This difference in species or cultivars' behavior is linked to a number of physiological and biochemical mechanisms developed by plants to survive, grow and produce in the presence of high salt concentrations including sodium ion exclusion, potassium ion maintenance and organic compounds accumulation. Among the compounds involved, there may be mentioned amino acids, in particular proline, soluble sugars, soluble proteins and quaternary ammonium compounds.

Okra (*Abelmoschus esculentus* L. Moench) is an annual vegetable of the tropical and subtropical areas belongs to family Malvaceae. It is a popular vegetable among both the consumers and farmers because it is rich in vitamins and minerals (Oyelade et al., 2003). Almost all parts of okra plant are consumed, like fresh okra fruits are used as vegetable, roots and stems are used for clearing the cane juice (Chauhan, 1972) and leaves and stems are used for making fiber and ropes (Jideani and Adetula, 1993). Being an excellent source of K, calcium (Ca) and unsaturated fatty acids for instance, linolenic and oleic acid (Arbona et al., 2005), okra is very essential for human nutrition (Khan et al., 2015). Although the area under okra has progressively increased during last few years, there is a decreasing trend in its yield per hectare (Haq et al., 2012). Among identified biotic and abiotic stresses, salinity has been the key factor responsible for yield reduction (Khan et al., 2002).

Despite a considerable amount of research work on plant responses to salt stress, data on okra salt tolerance strategy is scarce. In Asia, some research works studied the salt tolerance of some okra genotypes using several growth parameters (Haq et al., 2012 ; Abbas et al., 2014). Some other works studied the physiological strategy for salt tolerance in Asian okra genotypes (Shahid et al., 2011; Habib et al., 2012; Khan et al., 2015). In addition, there is hardly any work on the effect of salt stress on mineral nutrition, in particular on Na^+ and K^+ contents and on the accumulation of a number of organic compounds in okra cultivars grown in Benin Republic. In a previous study, it was found that an important variability exists among okra cultivars produced in Benin in term of salinity resistance (Gouveitcha et al., 2021). The main objective of this study was to determine the physiological strategy developped by salt resistant okra cultivars grown in Benin to resist, to salt stress related to ion and organic solutes accumulation.

MATERIAL AND METHODS

Plant

The experiment focused on two cultivars of okra (*Abelmoschus esculentus* L. Moench) which exhibited contrasting behaviors towards salinity at the young plant stage. These are the cultivars *Yodana* which appeared to be a resistant cultivar and *Keleya* which appeared to be sensitive to NaCl according to Gouveitcha et al. (2021).

Experimental conditions

The experiment was carried out in screening house of the National Institute of Agricultural Research of Benin (INRAB), Benin Republic. The seeds were germinated in tubs filled with potting soil for two weeks. The young plants were then transferred to small pots 5.8 cm in diameter and 6 cm in height containing a mixture of potting soil and sand (50:50) (one plant/pot) and grown for a week before application of the stress. Plants of the two cultivars were subjected to salt stress in large earthen pots (11.3 cm in diameter and 14 cm in height) filled with 3 kg of the same mixture as before. Treatments consisted of watering the plants every other day with 100 ml/pot of 0; 30; 60; 90 or 120 mM NaCl. The experimental set-up as a completely randomized design with two factors. The first factor represents the five (05) saline treatments (T0 = 0 mM; T1 = 30 mM; T2 = 60 mM ; T3 = 90 mM and T4 = 120 mM) and the 2nd is represented by the two (02) okra cultivars (*Yodana* and *Keleya*) with three replicates.

Growth determination

Plant height (cm), number of leaves, root length (cm), fresh and dry mass (FM and DM) of the shoots and roots were first determined before application of the salt treatments (X0). They were determined again after 2 weeks of treatment (X1). Relative height growth of plants (RHG) was determined according to the formula: (X1-X0) / X0. The fresh mass of the aerial and roots parts was determined by weighing. The samples from each part were then transferred to an oven at 80°C for 72 h for the determination of the dry mass. Data in the presence of NaCl were expressed in percentage of that of the control.

Extraction and estimation of ion concentrations

For the determination of the ions, the roots were quickly rinsed with distilled water to remove the ions fixed on them and those contained in the apoplasm (Bourgeais-Chaillou and Guerrier, 1992). The leaves and roots were individually dried in an oven at 80°C for 72 h, ground in a mortar, and the powder was dried for 24 h. To determine the concentrations of Na^+ and K^+, 20 mg of the leaf and root powders were placed in 10 ml jars and digested in nitric acid (68%) at room temperature. The solutions were filtered through Whatman paper (85 mm, Grade 1). The filtrate was used for the determination of cations (Na^+ and K^+) using a flame

Table 1. Effects of salt stress on some growth parameters of two okra cultivars after two weeks of treatment. Values are expressed as percentages (%) of means of control plants.

NaCl (mM)	Cv. *Keleya*					Cv. *Yodana*				
	RHG	RSFMG	RSDMG	RRFMG	RRDMG	RHG	RSFMG	RSDMG	RRFMG	RRDMG
30	94.63	81.00	78.60	83.04	75.78	95.94	92.02	93.31	62.83	72.57
60	81.30	69.92	75.66	69.68	75.06	93.07	84.87	77.05	59.71	66.71
90	75.31	53.47	65.37	56.89	59.92	87.93	66.41	69.95	44.90	47.43
120	68.10	16.19	13.71	39.18	47.05	70.35	59.10	54.21	38.98	40.02

spectrophotometer (Sherwood Model 360). The quantities of ions were expressed in mg g^{-1} of dry matter (dm).

Extraction and determination of proline and soluble sugars

Proline concentration was determined spectrophotometrically using the method of Bates et al. (1973) and results were expressed as µg proline g^{-1} FM (Fresh Mass).

Total soluble sugars were estimated by the anthrone reagent method using glucose as the standard accords to Yemm and Willis (1954) as used by Manaa et al. (2014) using an UV-visible spectrophotometer (Jenway 7305). Soluble sugars concentration was expressed as mg soluble sugars g^{-1} FM (Fresh Mass).

Shoot water content

Shoot water content was determined according to the formula:

[(Shoot fresh Mass – shoot dry Mass)/Shoot fresh Mass] × 100

Statistical analysis

The data collected was processed using descriptive statistics using an Excel spreadsheet and presented in the form of tables and graphs. Analysis of cultivar effects and stress intensity was based on one or two-ways analysis of variance (ANOVA) as appropriate. Means were compared using the Student, Newman and Keuls test. Statistical analyzes were performed using JMP Pro 12 software (JMP Pro SAS Institute, 2015).

RESULTS

Plant growth

NaCl stress significantly ($p< 0.001$) reduced RHG in both cultivars from 90 mM NaCl but the reduction was more accentuated in the salt-sensitive *Keleya* (average of 20.16%) than the salt resistant *Yodana* (average of 12.95%) (Table 1). Salt effect induced a reduction in shoot fresh and dry mass in both cultivars but the reduction was more accentuated in the salt sensitive cultivar *keleya* than the salt resistance *Yodana*. The average reductions due to NaCl stress were 44.85, 44.16%, for cultivar *Keleya* and only 24.40 and 26.37% for cultivar *Yodana*, respectively for shoot fresh mass and shoot dry mass (Table 1). Roots growth also was adversely affected by salt stress in both root fresh and dry mass in both cultivars. However, the reduction was less accentuated in the salt sensitive *Keleya* than the salt resistant *Yodana*. The average reductions due to NaCl stress were 37.80 and 35.55%, for cultivar *Keleya* and 48.39 and 43.32% for cultivar *Yodana*, respectively for roots fresh mass and roots dry mass (Table 1). Thus, plant aerial part growth reduction by NaCl stress was more accentuated in the salt-sensitive *Keleya* compared to the salt-resistant *Yodana*.

Plant water content

NaCl stress induced similar effect on shoot water content in both cultivars characterized by slight non significant decrease (Table 2). Thus, shoot water content did not change significantly under salt stress in both cultivars.

Effects of NaCl on ions accumulation

Effect of NaCl on sodium ion (Na^+) content

Salt effect induced a significant ($p< 0.001$) increase in the leaf Na^+ content of both cultivars but this increase was significant from 60 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant only at 120 mM NaCl for the resistant cultivar *Yodana* (Figure 1A). Leaf Na^+ content passed from 1.478 mg g^{-1} DM to 1.739, 2.369, 2.391 and 2.521 mg g^{-1} DM for cultivar *Keleya*, respectively at 30, 60, 90 and 120 mM NaCl and from 0.978 mg g^{-1} DM to 1.108, 1.26, 1.478 and 1.63 mg g^{-1} DM for cultivar *Yodana* at 30, 60, 90 and 120 mM NaCl, respectively. Thus, salinity induced an increase in sodium content in the leaves in both cultivars but this increase is more marked in sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana*. Likewise, Figure 1B shows a significant ($P< 0.01$) increase in the roots Na^+ content of both cultivars but this increase was significant from 30 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant from 90 mM NaCl for the resistant cultivar *Yodana*. Root Na^+ content passed from 1.956 mg g^{-1} DM to 2.673, 2.804, 3.195 and 3.347 mg g^{-1} DM for cultivar *Keleya*, respectively at 30, 60, 90 and 120 mM NaCl, and

Table 2. Effect of different NaCl concentrations on shoot water content (%) of two cultivars of *okra* after two weeks stress application.

NaCl (mM)	Cv. *Keleya*	Cv. *Yodana*
0	93.532±0.65[a]	94.198±0.42[a]
30	92.994±0.28[a]	93.860±0.27[a]
60	92.785±0.16[a]	93.901±0.27[a]
90	92.497±0.38[a]	93.731±0.30[a]
120	92.850±0.25[a]	93.468±0,24[a]

Means with same letter within column did not differ significantly at P=0.05.

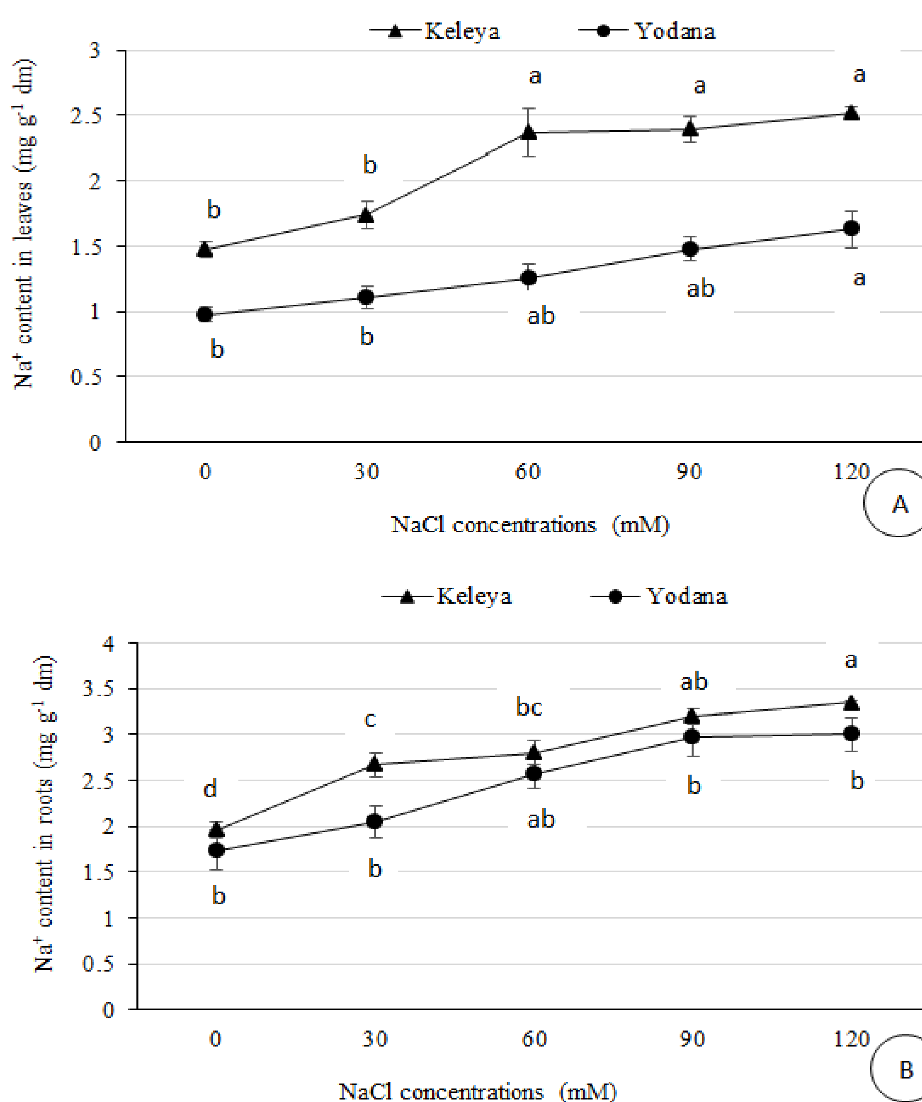

Figure 1. Effect of different NaCl concentrations on sodium ion content of two cultivars of *okra* after two weeks stress application (A) in leaves; (B) in roots (n= 3; vertical bars are standard errors). Means with different letters differ significantly at P=0.01.

from 1.739 to 2.043, 2.565, 2.978 and 3 mg g^{-1} DM for cultivar *Yodana* at 30, 60, 90 and 120 mM NaCl, respectively. Thus, salinity induced an increase in sodium content in roots in both cultivars but this increase is more marked in the sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana*.

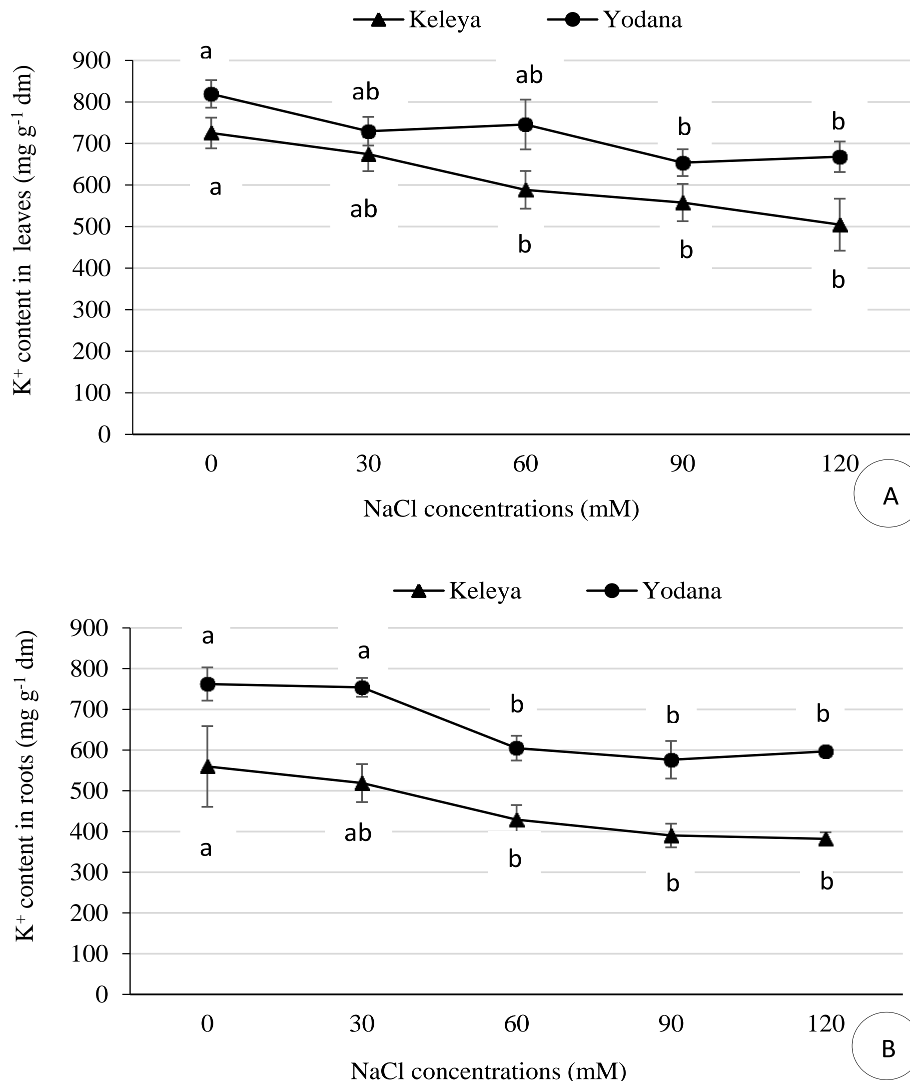

Figure 2. Effect of different NaCl concentrations on potassium ion content of two cultivars of *okra* after two weeks stress application (A) in leaves; (B) in roots (n= 3; vertical bars are standard errors). Means with different letters differ significantly at P=0.05.

Effect of NaCl on potassium ion (K^+) content

Salt effect induced a significant decrease in leaves K^+ content of both cultivars but this decrease was significant ($P<0.05$) from 60 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant ($P<0.01$) from 90 mM NaCl for the resistant cultivar *Yodana* (Figure 2A). Leaf potassium content passed from 725.37 mg g^{-1} DM to 674.29, 588.47, 557.82 and 504.7 mg g^{-1} DM for cultivar *Keleya*, respectively at 30, 60, 90 and 120 mM NaCl, and from 819.37 mg g^{-1} DM to 729.46, 745.81, 653.86, and 668.16 mg g^{-1} DM for cultivar *Yodana*. Thus, salinity induced a decrease in potassium content in leaves in both cultivars but this decrease was more marked in sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana*. Likewise, Figure 2B shows a significant ($p<0.05$) decrease in roots potassium content from 60 mM NaCl in K^+ content of both cultivars. Roots potassium content passes from 559.86 mg g^{-1} DM to 519, 429.10, 390.27, and 302.10 mg g^{-1} DM for cultivar *Keleya* and 762.16 mg g^{-1} DM to 753.98, 604.82, 576.22 and 596.65 mg g^{-1} DM for cultivar *Yodana*. Thus, salinity induced a decrease in potassium ion content in roots in both cultivars but this decrease was more marked in the sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana*.

The selectivity ratio decreased in both cultivars in leaves and roots as the NaCl concentration increased (Table 3). For the sensitive cultivar *Keleya*, the decrease was significant ($P<0.001$) from 60 mM NaCl. For the resistant cultivar *Yodana*, the decrease was significant ($P<0.01$) from 90 mM NaCl. Thus, salinity induced a

Table 3. Effect of different NaCl concentrations on selectivity ration (K/Na) in leaves and in roots of two cultivars of *okra* after two weeks stress application (n= 3; values are means ± standard errors).

NaCl (mM)	Leaves (K/Na)		Roots (K/Na)	
	Keleya	***Yodana***	***Keleya***	***Yodana***
0	491.64±23.50^{a}	844.63±54.31^{a}	291.56±57.62^{a}	456.29±55.74^{a}
30	390.36±24.91^{a}	666.90±30.71^{ab}	195.99±20.47^{b}	375.48±24.11^{ab}
60	251.93±24.91^{b}	615.80±12.13^{ab}	154.69±17.42^{b}	236.57±5.43^{b}
90	234.64±22.19^{b}	448.05±18.98^{b}	122.52±9.87^{c}	200.80±34.13^{c}
120	200.41±25.20^{b}	417.05±12.50^{b}	114.08±4.33^{c}	200.93±11.58^{c}

Means with same letter within column did not differ significantly at P=0.05.

decrease in K^+/Na^+ selectivity ratio of leaves for both cultivars but this decrease was more marked in a sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana*. In roots, the same tendency was observed with a decrease significant (P<0.001) from 30 mM NaCl for the sensitive cultivar *Keleya*, and significant (P<0.01) from 60 mM NaCl for the resistant cultivar *Yodana*.

Effect of salt stress on proline content

Salt effect induced a significant (P< 0.001) increase in proline content in leaves of both cultivars but this increase was significant (P< 0.01) only at 120 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant (p< 0.001) from 90 mM NaCl for the resistant cultivar *Yodana* (Figure 3A). Proline concentration passed from 0.018 µg g^{-1} FM to 0.021, 0.021, 0.026, and 0.036 µg g^{-1} FM for cultivar *Keleya*, respectively at 30, 60, 90, and 120 mM NaCl, and from 0.010 µg g^{-1} FM to 0.013, 0.018, 0.046, and 0.067 µg g^{-1} FM for cultivar *Yodana* at 30, 60, 90, and 120 mM NaCl, respectively. These increases correspond respectively to 16.66, 16.66, 44.44 and 100% compared to the control for the sensitive cultivar *Keleya*, and to 30, 80, 360, and 570% for the resistant cultivar *Yodana*. Thus, salinity induced an increase in proline content in leaves in both cultivars but this increase is much more marked in resistant cultivar *Yodana* compared to the sensitive cultivar *Keleya*. Likewise, Figure 3 shows a significant increase in roots proline content of both cultivars but this increase was significant (P< 0.01) only at 120 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant (P< 0.001) from 60 mM NaCl for the resistant cultivar *Yodana*. Proline concentration passed from 0.013 µg g^{-1} FM to 0.019, 0.016, 0.021 and 0.027 µg g^{-1} FM for cultivar *Keleya*, respectively at 30, 60, 90, and 120 mM NaCl; and from 0.005 µg g^{-1} FM to 0.008, 0.019, 0.043 and 0.049 µg g^{-1} FM for cultivar *Yodana* at 30, 60, 90, and 120 mM NaCl, respectively. These increases correspond, respectively to 46.15, 23.07, 61.53, and 107.69% compared to the control for the sensitive cultivar *Keleya*, and to 60, 280, 760, and 880% for the resistant cultivar *Yodana*. Thus, salinity induced an increase in proline content in roots in both cultivars but this increase is much more marked in the resistant cultivar *Yodana* compared to the sensitive cultivar *Keleya*. Moreover, proline accumulation was more marked in roots than leaves in both cultivars.

Effect of salt stress on soluble sugars content

Salt effect induced a significant (P< 0.001) increase in soluble sugars content in leaves of both cultivars but this increase was significant from 30 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant from 60 mM NaCl for the resistant cultivar *Yodana* (Figure 4A). Soluble sugars content passed from 0.441 mg g^{-1} FM to 0.73, 0.717, 1.059, and 1.804 mg g^{-1} FM for cultivar *Keleya*, respectively at 30, 60, 90 and 120 mM NaCl, and from 0.291 mg g^{-1} FM to 0.396, 0.529, 0.763, and 0.647 mg g^{-1} FM for cultivar *Yodana* at 30, 60, 90, and 120 mM NaCl, respectively. These increases correspond, respectively to 65.53, 62.58, 140.13 and 309.07% compared to the control for the sensitive cultivar *Keleya*, and to 36.08, 81.78, 162.19, and 122.33% for the resistant cultivar *Yodana*. Thus, salinity induced an increase in soluble sugars content in leaves in both cultivars but this increase is more marked in the salt sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana* mainly at 30 and 120 mM NaCl. Likewise, Figure 4B shows a significant (P< 0.001) increase in roots soluble sugars content of both cultivars but this increase was significant from 60 mM NaCl for the sensitive cultivar *Keleya* whereas it was significant from 90 mM NaCl for the resistant cultivar *Yodana*. Soluble sugars content passed from 0.215 mg g^{-1} FM to 0.224, 0.514, 0.655, and 0.994 mg g^{-1} FM for cultivar *Keleya*, respectively at 30, 60, 90 and 120 mM NaCl, and from 0.135 mg g^{-1} FM to 0.180, 0.174, 0.190 and 0.310 mg g^{-1} FM for cultivar *Yodana* at 30, 60, 90 and 120 mM NaCl, respectively This increase corresponds, respectively to4.18, 139.06, 204.65 and 362.32% compared to the control for the sensitive cultivar *Keleya*, and to 33.33,

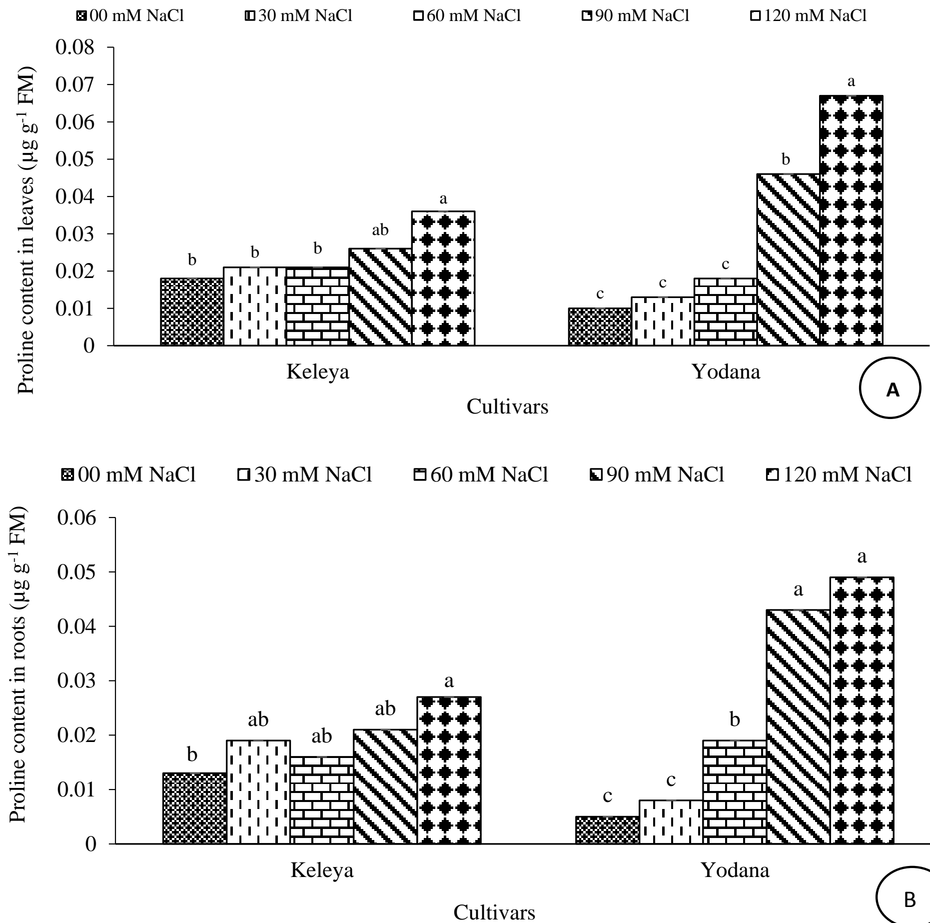

Figure 3. Effect of different NaCl concentrations on proline content of two cultivars of *okra* after two weeks stress application (A) in leaves; (B) in roots (n= 3 ; vertical bars are standard errors). Means with different letters differ significantly at P=0.001.

28.88, 40.74 and 129.62% for the resistant cultivar *Yodana*. Thus, salinity induced an increase in soluble sugars content in roots in both cultivars but this increase is much more marked in the sensitive cultivar *Keleya* compared to the resistant cultivar *Yodana* except at 30 mM NaCl.

DISCUSSION

Effect of NaCl on plant growth of okra cultivars

The results revealed that plant growth reduction due to NaCl stress was more accentuated in the salt-sensitive *Keleya* compared to the salt-resistant *Yodana* confirming the salt-resistance status of both cultivars as previously reported (Gouveitcha et al., 2021). In other okra genotypes, Abbas *et al.* (2014) used growth parameters to discriminate salt tolerant genotypes from the salt sensitive one. Salinity classically induced cell dehydration at low water potential, nutritional imbalance caused by the interference of saline ions with essential nutrients in both uptake and translocation processes and toxicity due to the high accumulation of Na^+ and Cl^- in the cytoplasm. No change in plant water content was observed in our cultivars indicating that water content parameter is not the main aspect of salt stress effect in these cultivars as previously reported in amaranth (Wouyou et al., 2019).

Implications of ion accumulation in the salinity resistance of okra cultivars

The effect of salt stress on plants can induce the following three responses: dehydration of cells through low water potential; nutritional imbalance caused by the interference of salt ions with essential nutrients in both absorption and translocation processes; toxicity due to a high accumulation of Na^+ in the cytoplasm and to a lesser extent of Cl^-. In most species, Na^+ appears to accumulate to toxic levels before Cl^- does (Negrão et al., 2017) and Cl^- is considered less toxic than Na^+ (Munns and Tester, 2008).

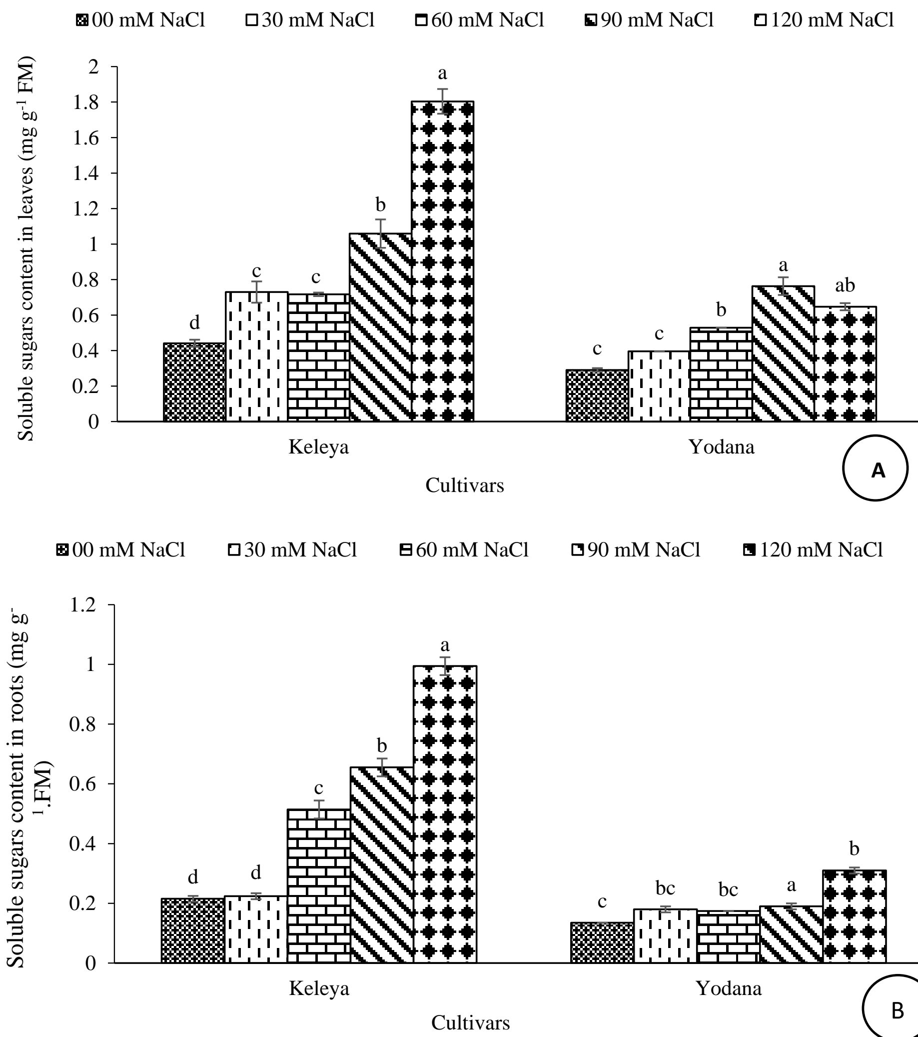

Figure 4. Effect of different NaCl concentrations on soluble sugars content of two cultivars of *okra* after two weeks stress application (A) in leaves; (B) in roots (n= 3; vertical bars are standard errors). Means with different letters differ significantly at P=0.05.

Thus, we focus here on Na^+, because reducing Na^+ in the shoot, while maintaining K^+ homeostasis, is a key component of salinity tolerance in many crops (Munns, 2011). Plants of both cultivars accumulated high amounts of Na^+ in the leaves when subjected to NaCl in their growing medium. The same tendency was reported in other okra genotypes (Habib et al., 2012). The results revealed that the resistant cultivar *Yodana* accumulated less Na^+ in the leaves and roots than the sensitive cultivar *Keleya*. However, it was observed that the harmful effects of Na^+ on growth are more accentuated on the aerial part of plants of the sensitive cultivar *Keleya*, compared to those of the resistant cultivar *Yodana*. Thus, the detrimental effects of Na^+ on growth are more accentuated in aerial part of the sensitive *Keleya* compared to cv. *Yodana* plants. This is a general trend in glycophytes in which salinity resistant varieties accumulate less Na^+ and/or Cl^- in the leaves than susceptible varieties (Lutts et al., 1996; Almansouri et al., 1999; Akhtar et al., 2003; Wahid, 2004; Niu et al., 2010; Wouyou et al., 2019). The relative resistance of the cultivar *Yodana* to NaCl is therefore explained by its ability to exclude Na^+ ions from the leaves leading to an avoidance or exclusion mechanism. In okra, Habib et al. (2012) have reported that foliar application of both pure glycine betaine and sugarbeet extract significantly reduced the adverse effects of salt stress in terms of plant growth, yield and leaf Na^+ content indicating that the salt tolerance acquired following the foliar application of both pure glycine betaine and sugarbeet extract was due, at least partially, to Na^+ exclusion from leaves. This result confirmed the importance of Na^+ exclusion from leaves as part of okra plants salt tolerance strategy.

Salt stress caused a decrease in potassium ion in the

leaves and roots of the two cultivars tested. This observation is also made by Maggio et al. (2007) who proved that the presence of NaCl in the plant environment generally induces an increase in Na^+ and a decrease in K^+ in the various organs. Similar results were reported in other okra genotype (Shahid et al., 2011). Maintaining a good K supply is one of the major responses of salt stress resistant genotypes in glycophyte species, and potassium ions are known to be a major component of osmotic adjustment during stress (Wu et al., 1996). Thus, in rice, Lutts et al. (1996) reported that a salt tolerant variety maintained high amounts of K^+ in the leaves compared to salt sensitive genotypes when both types were under salt stress. The same trend has been reported in durum wheat (Almansouri et al., 1999). In the present study, the reduction in potassium content observed is clearly less pronounced in the resistant cultivar *Yodana* compared to the sensitive cultivar *Keleya* in both leaves and roots. Thus, the relative resistance of cultivar *Yodana* to salinity appears to be primarily associated with maintaining a good K^+ supply in the presence of NaCl. Comparing the effect of foliar application of both pure glycine betaine and sugarbeet extract on okra response to salt stress, Habib et al. (2012) have revealed that this application significantly reduced the adverse effects of salt stress in terms of plant growth, yield and leaf K^+ content indicating that leaf K^+ accumulation is part of the strategy which mediated the salt tolerance acquired following the foliar application of both pure glycine betaine and sugarbeet extract. This result confirmed the importance of leaf K^+ accumulation as part of okra plants salt tolerance strategy.

The salt-resistant cultivar *Yodana* accumulated less Na^+ in both leaves and roots and maintained higher K^+ content than the salt-sensitive cultivar *Keleya*. Consequently, the salt-resistant *Yodana* maintained a significantly higher K^+/Na^+ selectivity ratio in both leaves and roots than the sensitive *Keleya*.

Implication of organic solutes accumulation in the salinity resistance of okra cultivars

Biosynthesis of osmoprotectants and compatible solutes are among the physiological principle and biochemical mechanisms developed by plants in order to survive in soils with high salt concentration (Gupta and Huang, 2014). Proline and soluble sugars are the main parts of these osmoprotectants and compatible solutes. Salinity caused an increase in proline content in both cultivars either in leaves or in roots. Accumulation of proline is frequently reported in plants subjected to salt stress (Mishra and Saxena, 2009; Bouassaba and Chougui, 2018). It has often been considered as a compatible osmoregulator which may be involved in the mechanisms of resistance to salt stress (Ehsanpour and Fatahian, 2003; Bouassaba and Chougui, 2018). Other functions have been suggested regarding the accumulation of proline in stressed tissues ; it could be: (1) a protective agent for enzymes and membranes (Van Rensburg et al., 1993; Solomon et al., 1994), (2) a free radical scavenger (Smirnoff and Cumbes, 1989), (3) a carbon and nitrogen storage compound (Jäger and Meyer, 1977) or (4) it could be involved in the regulation of cytosolic pH (Venekamp, 1989). However, results of the present study on stressed okra plants showed that the accumulation of proline accumulation is much more marked in resistant cultivar *Yodana* compared to the sensitive cultivar *Keleya* in both leaves and roots. We can therefore suggest that the overproduction of proline is okra plants response to salt stress and that the salinity resistance of the cultivar *Yodana* is associated with high proline accumulation. These results indicated that proline plays an important role in salinity tolerance as previously reported in several species (Watanabe et al., 2000; Mishra and Gupta, 2005). However, other authors have reported an opposite tendency in several species including sugar cane (Wahid, 2004; Gandonou et al., 2005, 2011), rice (Lutts and Guerrier, 1995; Lutts et al., 1996), tomato (Pérez-Alfocea et al., 1994) and amaranth (Wouyou et al., 2019). In other okra genotypes, Habib et al. (2012) have reported that foliar application of both pure glycine betaine and sugarbeet extract significantly reduced the adverse effects of salt stress in terms of plant growth and yield but reduced leaf proline in comparison to salt stress indicating that the salt tolerance acquired following the foliar application of both pure glycine betaine and sugarbeet extract was accompanied by a low leaf proline accumulation. This result suggested that proline hyper accumulation in leaves is not part of the strategy developed by okra treated plants to tolerate salt stress. Thus, the implication of proline in salinity tolerance in okra depends on the genotype and the type of salt-tolerance (genetic or artificial) taking into account.

In general, salinity caused an increase in soluble sugars content in both cultivars either in leaves or in roots. The results are consistent with the general trend. Indeed, the effects of salt stress generally result in an increase in the content of soluble sugars in both the leaves and the roots in several plant species (Bouassaba and Chougui, 2018; Wouyou et al., 2019) including okra (Abbas et al., 2014). Generally, the more tolerant cultivars accumulate more soluble sugars than the sensitive ones. Thus, Gandonou et al. (2011) reported that in sugarcane, the salinity resistant cultivar CP66-346 accumulates more soluble sugars in the leaves than the sensitive CP65-357 in the presence of salt stress. The same trend has been reported in calli, selected or not in this same species (Gandonou et al., 2005, 2006). On the other hand, in *Carthamus tinctorius*, Ashraf and Fatima (1995) reported that two salt-resistant accessions showed different responses: one accumulates more soluble sugars than sensitive accessions, while the other

resistant accession accumulates similar amounts of sugars as sensitive accessions, although it is more tolerant. Soluble sugars are known for their role in osmoregulation in plants exposed to osmotic stress. According to Cram (1976), among the osmotically active organic compounds, sugars contribute more than 50% of the total osmotic potential in glycophytes subjected to salt stress. The fact that the salt sensitive cultivar *Keleya* accumulated more soluble sugars in both leaves and roots than the salt resistant *Yodana*, seems to indicate that soluble sugars did not play an important role in cultivar *Yodana* salt resistance.

Conclusion

Salt stress caused an increase in sodium ions, free proline and soluble sugars content and a decrease in potassium ion content in both leaves and roots in both okra cultivars with a significant difference in the reaction of okra cultivars. The overall reaction of the cultivars indicates that the relative salt resistance of cultivar *Yodana* is reliable to a good sodium ion exclusion and a good potassium ion accumulation mainly in leaves associated with maintaining of a good K^+/Na^+ ratio. Free proline appears also as a good salt resistance indicator in this cultivar.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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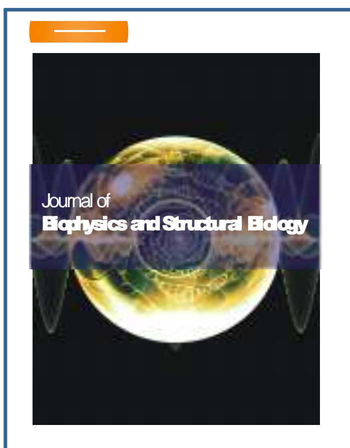

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