INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print), 2222-3045 (Online)
http://www.innspub.net
Vol. 6, No. 3, p. 238-247, 2015

RESEARCH PAPER | OPEN ACCESS

Response of *Stevia rebaudiana* Bertoni root system to waterlogging and terminal drought stress

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Key words: Stevia; Root Surface Area Density; Root volume and hypoxi.

Article published on March 17, 2015

Abstract

This research studied the effect of waterlogging level and duration, nitrogen fertilizer and terminal drought stress on root and shoot growth of Stevia. Treatments included four waterlogging levels; (0, -5, -10 cm from soil surface and with normal irrigation as control), in periods of 2 and 4 days of waterlogging, two levels of nitrogen (6‰ and 0) from source of urea and two levels of terminal drought stress. Results showed measured root and shoot traits include root dry weight, root length, root area, root volume, root length density, root surface area density, dry root mass density, root diameter, leaf area, plant height, number of SPAD, number of lateral shoot, number of leaves were significantly different among treatments. Root dry weight, root length and root volume in -10 waterlogging level treatment(16.24 g, 24.8 cm and 27.7 ml, respectively) and in fertilizer treatment (17.9 g, 25.5 cm and 22.1 ml, respectively) shown the least decrease. Moreover, interaction between drought stress treatment and nitrogen fertilizer for root dry weight and root length (18.4 g and 26.5 cm, respectively)and in shoot traits, number of leaves, leaf area and plant height (237, 64.07 cm^2 and 87 cm) showed the minimum difference with control treatments. Generally, the results of this experiment showed that increase of waterlogging either level or duration decrease the amount of root and shoot characteristics. Nitrogen fertilizer resulted in higher root and shoot traits in applying treatment. By contrast, terminal drought stress led to decrease in all root and shoot traits.

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Introduction

Stevia rebaudiana B. is a branched succulent shrub of the Asteraceae family, native to the Amambay region in the northeast of Paraguay (Soejarto, 2002). Presently, Stevia is well-known for its high content of sweet diterpene (C10) (about 4–20%) in dry-leaf matter (Ghanta *Et. al.* 2007). It is the source of a number of sweet ent-kaurene diterpenoid glycosides (Prakash *Et. al.* 2008).

In waterlogged soils carbon dioxide, ethylene, manganese, and iron compounds may accumulate (Ponnamperuma, 1984) in concentration potentially toxic to plants. However, oxygen deficiency (hypoxia) is the most important cause of flooding injury (Kozlowski, 1997).

Heavy textured soils in northern regions of Iran are more susceptible to waterlogging, and hypoxia is highly likely to limit root growth due to alternation in metabolism if there is <10% air-filled pore space (Dasilva *Et. al.* 1994). Thus waterlogging is considered as one of the important limiting factors for Stevia cultivation in such regions. Low amount of German Chamomile (*Matricariachamomilla L.*) essential oil yield at 100% field capacity could be attributed to excess water as this condition may reduce oxygen supply to the roots (Pirzad *Et. al.* 2006).On the other side, in northern areas of Iran the crop is planted after the main rainy season and grown on stored soil moisture making terminal drought stress a primary constraint on productivity, so may terminal drought stress limit leaf yield of Stevia. In chickpea, the focus of drought tolerance is on the ability to sustain greater biomass production and crop yield under a seasonally increasing water deficit, rather than the physiological aptitude for plant survival under extreme drought shock (Serraj and Sinclair 2002). This has led to the focus on escape and avoidance strategies, such as early maturity (Kumar, 1986) and large root systems (Singh and Saxena 1990). Field studies in various crops showed that both dense root systems extracting more water in upper soil layers and longer root systems extracting soil moisture from deeper soil layers are important for maintaining yield under terminal drought stress (Saxena and Johansen, 1999).

The objectives of the present experiment were to investigate the effects of waterlogging stress, nitrogen fertilizer and terminal drought stress on root and shoot traits of Stevia.

Materials and methods

Experimental design

Plant materials grew during 2013 growth season at the research farm of University of Guilan, Rasht , Iran (49° 39ʹE, 37° 12ʹ E, and 12 m below sea level) (Nabavi-Pelesaraei *Et. al.* 2014).

A screenhouse pot experiment was conducted in University of Guilan in spring, 2013. The experimental design was factorial with completely randomized design layout with three replications. Treatments included four waterlogging levels; (0, -5, -10 cm from soil surface and with normal irrigation as control), in periods of 2 and 4 days of waterlogging, two levels of nitrogen (6‰ and 0) from source of urea and two levels of terminal drought stress.

Each experimental unit (pot) contained PVC tubes of 50 cm in length and 16 cm in diameter and the Stevia seedlings with 3 plants in each pot were planted in equal distance. Before running the experiment, soil physical and chemical properties and macro elements were determined.

Large bins with a height of 50 cm and a diameter of 80 cm were filled with water in which PVC tubes containing Stevia plants were accommodated inside them for modeling waterlogging stress (Fig. 1).

Treatments

Waterlogging stress was applied in early stage of plant growth, when opposite side shoots began growth, after that, nitrogen fertilizer was sprayed (0.75 mg in 1000) by manual sprayer on plants in the evening so plants did not burnt. Spraying was continued till the drops of soluble fertilizer dripped down the leaves. However, for drought stress, two weeks before harvest, relative water content of sample leaves were measured and only if it reached 15% lower than the control plants, irrigation was applied. Climate data related to research farm of University of Guilan, which gathered from Guilan Province Meteorological Organization, is available in Table 1.

Table 1. Climatic information of the experimental site during drought stress treatment in 2013.

2013	Average of Maximum temperature (°C)	Average of minimum temperature (°C)	Average of Maximum humidity (%)	Average of minimum humidity (%)	Average of humidity (%)	Rainfall (mm)	Evaporation (mm)
September	28.5	20.5	98	68	83	66.7	88.6

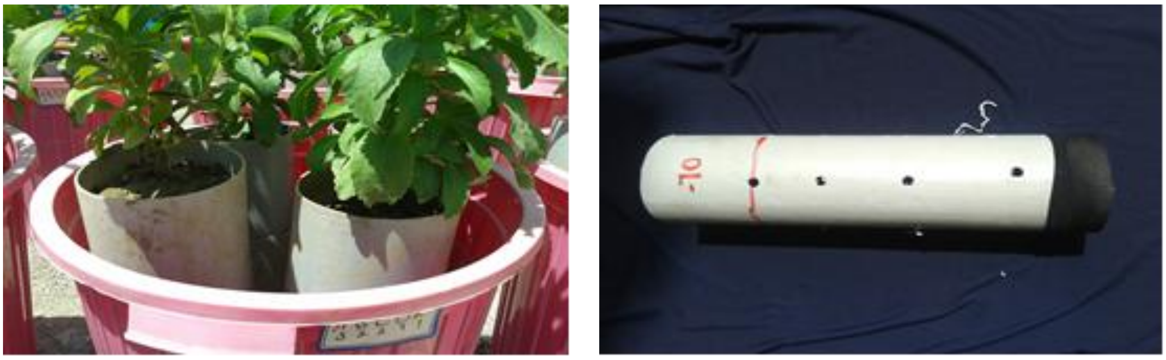

Fig.1. Pot and PVC tubes used for waterlogging stress impose on stevia plants.

Root studying

For root traits studies, at harvest time, the pots were flooded for one hour to wash the roots with minimal damage. Then data for tap root length (TL) (root weight) × 0.89 , root volume, root fresh weight (RFW), root dry weight (RDW), root area (RA) by Atkinson procedure = 2(root volume×π×root length)$^{0.5}$, root dry weight / root volume (RDW/RV), root mass density (RMD), dry root mass density (DRMD), root length density (RLD),root diameter (RD) =(4*RFW/(RL*3.14))1/2, root length / root fresh mass= (RF), root surface area density (RSD) (TL*RD*3/14) were measured (Hajabbasi 2001; Ganjali *Et. al.* 2003).

Shoot studying

Shoot traits were measured 120 days after planting before flowering phase these traits include: leaf area (LA) by leaf area measurement device (Li Core3100, USA), plant height, chlorophyll content by manual chlorophyll measurement (SPAD502 Minolta, Japan) were measured. The number of lateral shoots the number of leaves on main and lateral shoots were counted.

Statistical analysis

The data were subjected to analyses of variances (ANOVA) using SAS computer package (SAS version 9.2). Means comparisons were conducted using Fisher's (protected) Least Significant Differences (LSD).

Results and discussion

Root traits

Analysis of variances indicated significant differences among treatments for most of the traits. Waterlogging level, waterlogging duration, applying nitrogen fertilizer had a significant effect on RDW, RL, RA, RV, RLD, RSD, DRMD. In addition, Terminal drought stress except for RDW and RA had the same effect on other traits.

Means shown in Table 2 declared that waterlogging stress decreased root traits in Stevia. The highest value of RL and RA were recorded in -10 cm level of waterlogging (24.8 cm, 84.5 cm^2 respectively). There was no significant difference between RDW and RA in -5 and -10 waterlogging level. Moreover, the highest values for RLD, RSD and RD were observed in -10 waterlogging level (30.94 $cm.cm^{-3}$, 114.9 cm^2cm^{-3}, and 1.47 mm, respectively). In lower waterlogging level the more value of traits were observed. In waterlogging duration treatment all traits values for 4 days waterlogging were significantly different from those for 2 days except RDW. The values of all traits measured in a four-day period were lower than those measured in a two-day period (Table 2).

Nitrogen fertilizer led to significant increase among all traits in comparison with control (Table 2). Under 2 days waterlogging duration, applying nitrogen fertilizer, increased root dry weight (18.4 g), root length (26.5 cm), root area (87.1 cm^2), RLD (0.706 $cm.cm^{-1}$), RSD (129$cm^2.cm^{-3}$), DRMD (23.03 $g.cm^{-3}$) and root diameter (1.51 mm) (Table 3). Root volume, by contrast, was not affected by nitrogen fertilizer and showed lower value (20 ml) comparing to the other treatment (22.9 ml) where the nitrogen fertilizer was not applied. In 4-day waterlogging treatment application of nitrogen led to an increase in rate of above mentioned traits except root dry weight (12.9 g).

Table 2. Mean root characteristics under waterlogging levels and durations, nitrogen.

Treatments	Levels (cm)	Root Dry Weight (RDW) (g)	Root length (cm)	Root area (cm^2)	Root volume (ml)	Root Length Density (RLD) ($cm.cm^{-1}$)	Root Surface Area Density (RSD) ($cm^2.cm^{-3}$)	Dry Root Mass Density (DRMD) ($g.cm^{-3}$)	Root diameter (mm)
Waterlogging	0	10.9c	17.2d	56.1d	14.7c	21.5d	65.9d	13.6c	1.21d
	-5	14.1b	20.2c	69.5c	19.1b	25.1c	81.2c	17.5b	1.27c
	-10	16.2b	24.8b	84.5b	22.8b	30.9b	114b	20.2b	1.47b
	Control	22.1a	33.79a	99.60a	27.70a	42.03a	202.75a	27.54a	1.91a
Waterloggin duration	2 days	16.5a	25a	81.8a	21.4a	31.1a	121.6a	20.5a	1.49a
	4 days	15.1a	23b	73.08b	19.2b	28.7b	110.8b	18.9b	1.44b
Nitrogen Fertilizer	6‰	17.9a	25.5a	82.8a	22.1a	31.7a	123a	22.3a	1.48a
	0	13.7b	22.6b	72.1b	18.5b	28.1b	108b	17.1b	1.45a
Drought stress	Stress	16.03a	22.8b	77.4a	21.7a	28.4b	113.1b	19.9a	1.49a
	Control	15.6a	25.2a	77.4a	19.02b	31.4a	119.3a	19.5a	1.43b

applicationand terminal season drought stress in stevia
Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Interestingly, under drought condition RL and RV (25.27 cm, 21.7 ml, respectively) were more than in non-stress condition (22.83 cm, 19.2 ml, respectively). While, other root traits like all of shoot ones had less values under drought stress compare to non-stress conditions.

The value of Root Length (26.5 cm) and Root Length Density (0.708 $cm.cm^{-1}$) under drought stress were more in comparison with control (24.4 cm and 0.650 $cm.cm^{-1}$, respectively). However, in nitrogen applied treatments the amounts of Root Volume (23.1 ml), Dry Root Mass Density (21.7 $g.cm^{-3}$), and Root Diameter (1.52 mm) in control were more than stress treatment (21.2 ml, 22.9 $g.cm^{-3}$ and 1.43 mm, respectively). No difference was observed between the amount of Root Surface Area and Root Area.

In none applying nitrogen condition, values of Root Length (23.9 cm), Root Length Density (0.638 $cm.cm^{-1}$), and Root Surface Area Density (113 $cm^2.cm^{-3}$) were more than control treatment (21.5 cm, 0.566$cm.cm^{-1}$, and 104 $cm^2.cm^{-3}$ respectively) while, Root Volume (20.2 ml) and Dry Root Mass Density (18.1 $g.cm^{-3}$) showed higher values in control condition. No Significant differences were seen between stress and control treatments in Root Diameter, Root Area, and Root dry Weight of Stevia plants.

In drought stress treatment, however, differences for all measurementsweresignificant in favour of control treatment except for RDW, RA, and DRMD in which no significant differences were observed.

Table 3. The effect of nitrogen level on the response of root traits to waterlogging level in stevia.

Waterlogging duration (Days)	Nitrogen level	Root Dry Weight (RDW) (g)	Root length (cm)	Root area (cm^2)	Root volume (ml)	Root Length Density (RLD) ($cm.cm^{-1}$)	Root Surface Area Density (RSD) ($cm^2.cm^{-3}$)	Dry Root Mass Density (DRMD) ($g.cm^{-3}$)	Root diameter (mm)
2 days	0	14.5c	23.5c	76.5b	22.9a	0.626c	113b	18.1c	1.47ab
	6‰	18.4a	26.5a	87.1a	20b	0.706a	129a	23.03a	1.51a
4 days	0	12.9d	21.7d	67.6c	17.1c	0.578d	104c	16.1d	1.43b
	6‰	12.9d	24.5b	78.4b	21.4ab	0.653b	117b	21.7b	1.45b

Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Table 4. The effect of nitrogen level on the response of root traits to drought level in stevia.

Nitrogen level	Drought level	Root Dry Weight (RDW) (g)	Root length (cm)	Root area (cm^2)	Root volume (ml)	Root Length Density (RLD) ($cm.cm^{-1}$)	Root Surface Area Density (RSD) ($cm^2.cm^{-3}$)	Dry Root Mass Density (DRMD) ($g.cm^{-3}$)	Root diameter (mm)
6%	Stress	18.4a	26.5a	83.9a	21.2b	0.708a	125a	22.9b	1.43b
	Control	17.4a	24.4b	81.6a	23.1a	0.650b	122a	21.7a	1.52a
0	Stress	12.9b	23.9b	73.2b	16.8c	0.638b	113b	16.09c	1.43b
	Control	14.5b	21.5c	70.9b	20.2b	0.566c	104c	18.1b	1.47b

Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Shoot traits

The effects of experimental treatments on leaf area, plant height, number of lateral shoot, number of SPAD and number of leaves were significant.

Means shown in Table 5 said that waterlogging stress lessened shoot traits in Stevia. The lowest value of number of SPAD, lateral shoot and number of leaves were recorded in full waterlogged treatment (33.9, 18.5, and 217.8 respectively). Moreover, the lowest values for leaf area and plant height were observed in -5 waterlogging level (41.4 cm^2, and 76.6 cm respectively) (Table 5). In waterlogging duration treatment all traits values for 4-day waterlogging were different considerably from those for 2 days. The values of all traits measured in a four-day period were lower than those measured in a two-day period (Table 5).

Under 2 days waterlogging duration, applying nitrogen fertilizer, increased all shoot traits except plant height (104.5 cm) (Table 6). In 4-day waterlogging treatment application of nitrogen led to an increase in rate of leaf area, plant height and number of SPAD (55.7 cm^2, 127cm, and 39.5 respectively).

Table 5. Mean shoot characteristics under different flooding levels and duration, nitrogen application and end season drought stress in stevia.

Treatments	Levels (cm)	Leaf area (cm^2)	Plant Height (cm)	SPAD value	Number of lateral shoot	Number of leaves
Waterlogging level	0	49.4c	79.9c	33.9d	18.5d	217.8d
	-5	41.4d	74.6d	39.9c	20.2c	227.8c
	-10	64.07b	87.9b	43b	22.7b	237.6b
	Control	71.02a	95.5a	45.2a	31.2a	262.3a
Duration	2 days	58.2a	79.3b	41.2a	24.1a	239.6a
	4 days	54.7b	89.6a	39.8b	22.3b	233.08b
Fertilizer level	6‰	48b	53.1b	41.4a	21.9b	236.7a
	0	57.1a	115.9a	39.7b	24.5a	236.06a
Drought	Stress	239a	28.8a	40.2b	24.8a	239.08a
	Control	52.7b	81.7b	43.1a	31.5b	233b

Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Table 6. The effect of nitrogen level on the response of shoot traits to waterlogging level in stevia.

Waterloggin duration (day)	Nitrogen level	Leaf area (cm^2)	Plant Height (cm)	Number of SPAD	Number of lateral shoot	Number of leaves
2 days	0	57.9ab	54.1c	41a	23.08b	239a
	6‰	58.5a	104b	40.1a	21.5a	241a
4 days	0	53.8b	52.08c	39.2b	20.7c	233ab
	6‰	55.7ab	127a	39.5ab	23.8b	232b

Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Table 7. The effect of nitrogen level on the response of shoot traits to drought level in stevia.

Nitrogen level	Drought level	Leaf area (cm^2)	Plant Height (cm)	Number of SPAD	Number of lateral shoot	Number of leaves
0	Control	59.4a	55.5c	41.3ab	23.2b	238ab
	Stress	52.2b	50.6d	39.6c	20.6c	234ab
6%	Control	60.9a	118a	42.3a	26.4a	239a
	Stress	53.3b	112b	40.5b	22.5b	232b

Means in each column followed by similar letters are not significantly different at 5% probability level, using LSD test.

Discussion

According to the results of this experiment, waterloggingstress has negative effects on the most root traits measured in stevia plants, including RDW, RL, RA, RV, RLD, RSD and DRMD and all shoot traits. These results confirm the first hypothesis that waterloggingdecreases normal growth of *S. rebaudiana*root and shoot and consequently allowing it to survive.

Deprivation of oxygen to the roots of plants is the mainconsequence of waterlogging. In most cases, oxygen shortage directly affects roots. This condition may reduce oxygen supply to the roots, which in turn limits respiration, nutrient uptake and other critical root functions (Hopkins and Hunner 1995). Plants under waterlogging stress exhibit growth reduction and decrease in biomass production when compared to their well-watered counterparts (Kozlowski 1997; Li and Li 2005). Thus, our results are in agreement with the assumption of Zeng *Et. al.* (1999), who considered that sacrifice of non-essential sinks such as root may be advantageous to survival under the extreme conditions imposed by waterlogging or flooding, and with the finding of Kozlowski (1997) who demonstrated that an excess of soil water affects root growth. Under waterlogging conditions some species develop a system of adventitious roots in order to contribute to plant oxygenation, the same is true about *Stevia rebaudiana* (Visser *Et. al.* 1996; Thomas 1997; Li and Li 2005; Reis *Et. al.* 2011).

Interaction of waterlogging duration and nitrogen on chlorophyll content show reduction of the chlorophyll content (Table 6), that is because of the decrease in synthesis rate or break and rapid demolition of chlorophyll molecules. Gonzales and *Et. al.* (2009) who studied physiological responses of quinoa(*Chenopodium quinoa* Willd.), observed a significant decrease in LA in the waterlogging treatment in comparison with those under well-watered conditions. By contrast, under drought a smaller decrease in LA values was observed. The same results occurred in the present experiment.

In addition, several authors have proposed that leaf number can be used to characterise plant assimilation capacity (Hoogenboom *Et. al.* 1987; Gonzales *Et. al.* 2009). In the present study, also difference in this parameter was observed.

In this experiment, application of soluble nitrogen fertilizer (0.75 in 1000) dry soil partially recovered negativeeffects of waterlogging stress on all measured traits. Sincethe waterlogging stress increases nitrogen leaching insoil, therefore results of this study can be referring tothe effect of nitrogen fertilizer in preventing the nitrogen deficit. Fan *Et. al.* (2005) indicated that wheat leaf photosynthesis could be regulated by nitrogen supply under stress of waterlogging. Gusewell *Et. al.* (2003) observed that flooded plants were as nutrient-limited in the field, interactions between nutrient supply and water regime may be mediated by the effects of flooding.

Results of the experiments have indicated that nitrogen fertilizer, play a main role in deferral leaves wilting and yellowing under waterlogging stress since, nitrogen deficit leads to translocation of nitrogen from old leaves to young leaves (Zhou *Et. al.* 1997; Hodgson 1982). It seems that foliar application of nitrogen fertilizer after waterlogging stage alleviated plant damage caused by waterlogging by retarding chlorophyll and nitrogen degradation. Bacanamwo *Et. al.* (1997) found that recovery of N2 fixation is coincident with adventitious root formation as commented earlier. The waterlogging-induced N deficiency may be the cause ofthe decreased biomass accumulation in roots as our results showed that values for DRMD after waterlogging treatment but not applying N was less than application treatment (Table 3). Therefore, besides draining off water, alleviation of waterlogging damage may be controlled by applying nitrogen fertilizer.

By contrast, under drought a smaller decrease in RDW values were observed since decrease in RDW have been reported as a common effect of drought stress (Lawlor and Leach 1985), as in this condition plants for more water uptake produce more root. Stevia plants under similar conditions do not exhibit a significant reduction in RA and DRMD (Table 2). Under terminal drought, slow use of water (conservative strategy) is beneficial if the effective root depth is limited. Total root conductivity which is the inverse of root resistance ($Kr = 1/Rr$) is positively related to root length density in the soil and the hydraulic conductivity of the single root axe. High root length density increases the number of contact points between root and soil. This is crucial for water uptake in a drying soil.

Claimed that drought reduced the number of green leaves in potato cultivars these results are in close agreement with those of present experiment. It was found that increasing levels of water stress reduce growth and yield due to reduction in photosynthesis and plant biomass. Under increasing water-stress levels photosynthesis was limited by low CO_2 availability due to reduced stomatal conductance. Drought stress is associated with stomatal closure and thereby with decreased CO_2 fixation (Khalid 2006; Leithy *Et. al.* 2006). Interaction between terminal drought stress and nitrogen fertilizer showed an increase in almost all of root traits which had been received nitrogen. Low nitrogen supply and drought stress both reduced stevia growth and increased dry matter allocation to roots as differences between shoot traits which received nitrogen or did not, were smaller in comparison with root ones. Drought stress increased root length. The amount of this increase was more when nitrogen was applied.

Generally, these interactions showed that application of nitrogen fertilizer is likely that leads to reduce the negative effects of waterlogging or drought stress. Nitrogen also leads plants favor root growth over shoot growth under stresses and the root/shoot ratio increases.

Conclusion

Based on these results, can be concluded that increase of waterlogging either level or duration decrease the amount of root and shoot characteristics, in which control and two days length have the highest value in all root and shoot traits. Nitrogen fertilizer was optimum for plant production and root growth, resulting in higher root and shoot traits in applying treatment. By contrast, terminal drought stress led to decrease in all root and shoot traits. Although, nitrogen treatment helped to cut down the damage of terminal drought stress in all shoot and root traits. It seems the recovery role of nitrogen is because of its important physiological turn which is an essential component of all enzymes and therefore necessary for plant growth and development. Moreover, since nitrogen uptake, biomass production, and yield are strongly correlated any interruption in nitrogen requirement of crops causes a decrease in root/shoot traits.

For further studies, as waterlogging affects nitrogen movement in the xylem of stevia, it is recommended for analysis to find out which alterations may occur in nitrogen metabolism pathway under waterlogging stress.

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