Investigation Reducing Detrimental Effects of Salt Stress on Morphological and Physiological Traits of (*Thymus vulgaris*) by Application of Salicylic Acid

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Abstract

Salicylic acid (SA) is a naturally occurring plant hormone that has positive effects on growth and tolerance to biotic and abiotic stresses, especially salinity in plants. To evaluate the effects of SA and salt stress on some morphological and physiological traits and quantitative activities of antioxidant enzymes on thyme (*Thymus vulgaris*), was conducted a factorial pot experiment based on completely randomized design with four levels of SA (0t 150, 300 and 400 ppm) and four levels of salinity stress (0, 50, 100 and 150 mM, induced by NaCl) and three replicates. The results showed that salinity increased soluble sugars, quantitative activities of peroxidase and fresh weight and decreased stem height, leaf area and protein content. Interaction effects between salt stress and SA treatments resulted to greater leaf area, fresh weight, protein content and quantitative activities of catalase and peroxidase, significantly. Based the results of this study, leaf sparing of SA, improve physiological traits and alleviate salt stress effects through influencing physiological processes as increasing of antioxidant activity enzymes and soluble sugars content in *Thymus vulgaris*.

Keywords: Antioxidant enzymes; *Thymeus vulgaris*; salt stress; growth regulators; vegetative traits

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Introduction

Salinity is one of the important environmental stresses limiting soil fertility that adversely effects on growth and productivity of plants around the world (Khan and Panda, 2008; Parviz and Satyawati, 2008). More than 800 million hectares of lands throughout the world are salt-affected (including both saline and sodic soils), equating to more than 6% of the world's total land area. Iran, Pakistan, Egypt, and Argentina are salt affected regions that about 23.8, 10, 8.7, and 33.1 million hectares of their total lands are salty, respectively (FAO, 2010; FAO**,** 2008).

In order to adapt with the changes taking place in their environment, initially plants perceive environmental stresses and activate a range of defensive mechanisms (Sticher *et al.* 1997). One of the most obvious changes in plant response to increasing soil salinity is stopping of vegetative growth and leaf development (Al-whaibi *et al*., 2011). The earliest response is a reduction in the rate of leaf surface expansion followed by

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cessation of expansion as the stress intensifies but growth resumes when the stress is relieved (Parida and Das 2005). Changes in plant growth are due to the deleterious effects of salinity on physiological and biochemical mechanisms, which lead to Ion toxicity, osmotic and oxidative stress (Frary *et al*., 2010). The plants can tolerate salinity by multiple biochemical pathways that facilitate retention and/or acquisition of water, protect chloroplast functions and maintain ion homeostasis. Main pathways for development of tolerance are synthesis of osmotically active metabolites, specific proteins and certain free radical enzymes to control ion and water flux and support scavenging of oxygen radicals (Parvaiz and Satyawati, 2008). The key processes such as photosynthesis, protein synthesis and energy and lipid metabolisms are affected by the onset and development of salt stress within a plant (Parida and Das, 2005).

On the other hand, all environmental stresses can lead to the production of reactive oxygen species (ROS) that cause oxidative damages. The plants with high levels of antioxidants have more tolerance against oxidative damages (Said-Alahl and Omer, 2011). Reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide (O^{2-}), hydroxyl radical (HO^-) and singlet oxygen, can disrupt normal plant metabolism through oxidative damage to proteins, lipids, nucleic acids, photosynthetic enzymes and pigments (Ghasemzadeh and Jaafar, 2013). Under salinity stress, the activity of antioxidant enzymes increased in plants (Said-Alahl and Omer, 2011). Defensive mechanisms may also be induced or enhanced by the application of some chemicals to the plants (Raskin, 1995; Rajasekaran and Blake, 1999).

Signaling compounds that are able to reduce the adverse effects of stresses on plants could be of great importance to restoration of natural ecosystems as well as agricultural, horticultural and forestry production systems (Najafian *et al*., 2009). Salicylic acid (SA) is a group of plant growth regulator and its role in the defense mechanism is reported by many researchers in plants (Hussain *et al*., 2010). It has been reported that, Salicylic acid may induce tolerance to some of the major environmental stresses including chilling (Senaratna *et al.,* 2003) salt (Najafian *et al*., 2009) and drought (Senaratna *et al.* 2003). Exogenous application of SA has been shown to influence a range of diverse processes in plants, including seed germination, stomatal closure, ion uptake and transport, membrane permeability, photosynthesis, and plant growth rate (Aftab *et al*., 2010).

Thyme (*Thymus vulgaris* L.) belonging to the lamiaceae family is an important medicinal plant, which grows in several regions in the world (Davis, 1982). It has been used for centuries as spice, home remedy, drug, perfume and insecticide. Thyme also possesses various beneficial effects as antispasmolytic, antibacterial, antifungal, secrotolytic, expectorant, antiseptic, antlelmintic and antitusive, antiseptic, carminative, antimicrobial and antioxidative properties (Ozguven and Tansi, 1998; Baranauskiene *et al*, 2003).

The leaves of *Thymus vulgaris* are used as an herb in food preparations ،while the essential oil extracted from the leaves is used in beverages and the cosmetic and pharmaceutical industries (Naghdi badi and Makkizadeh, 2003).

The results of Hoseini (2010) in *Thymus vulgaris* were shown that with increasing of salt stress levels the content of proline and soluble sugars were increased, significantly. In other study was reported that Salicylic acid application in *Thymus vulgaris* was increased shoot and root dry weights compared to untreated plants when exposed to salt stress (Cik, 2009; Najafian *et al*., 2009).

The present study was undertaken to determine the effects of salt stress in the growth, physiological traits, and quantitative activity of antioxidant enzymes of *Thymus vulgaris* and to explore the role of Salicylic acid in salt stress mitigation in this regard.

Materials and Methods

This research was conducted in Islamic Azad University of Damghan branch, in 2012. The experimental design was two factors factorial, arranged in a completely randomized design, with three replications. The first factor was salinity stress (0 for control, 50, 100 and 150 mM of NaCl solutions) and the second factor was salicylic acid

treatment (0, 150, 300 and 450 ppm) as spray application.

The seeds of *Thymus vulgaris* were planted into plastic pots (25cm in length and 30 cm in diameter), which were filled with sandy loam soil, pH: 7.74, and kept under light/dark cycle conditions of 16/8 h at 23 °C and 75% relative humidity placed in a glass greenhouse. Four weeks after seed planting, thyme seedlings were subjected to 0 mM (control), 50 mM, 100 mM and 150 mM NaCl concentrations at 5 day intervals using 0.5 L irrigation water per pot. One week after starting salt stress treatment, SA was applied on the foliage of *Thymus vulgaris* plants with a hand sprayer, which occurred after covering the soil surface in order to omission of SA interfering via soil. This allowed for a known amount of SA for plant uptake. All plants were harvested 30 day after starting of treatments and separated into leaves, stem, and root.

Morphological traits

All plants were harvested 60 day after planting (30 days after treatments) and some morphological traits including plant fresh and dry weight, stem height, root/stem ratio and leaf area (Leaf Area Meter model ΔT England), were measured.

Biochemical assay

Total soluble sugar was determined according to modified method described by Eshligel (1986). Also, protein content (Lowry *et al*., 1951) and quantitative activities of antioxidant enzymes as catalase (Eising and Gerhardt, 1989) and peroxidase (Chance and Maehly, 1955) were determined.

Statistical analysis

The statistical analysis was performed using Microsoft Excel (2007) and SAS software and means were compared using Duncan's multiple range test at $\alpha = 0.05$.

Results

Stem height

As shown in Table 1, different levels of NaCl ($P \leq 0.01$) and interaction between NaCl × SA ($P \leq 0.05$), had a significant effect on stem height but SA treatment had no significant effect. Comparison of means shows that with increasing of salinity levels, stem height decreased. The highest and the lowest stem height were observed in 0 and 150 mM of NaCl, respectively (Table 2). Mean comparison of Interaction between NaCl × SA was showed that stem height was increased by SA treatment under salt stress, as the highest stem height was observed in 50mM NaCl × 150 ppm SA.

Root/stem ratio

The results showed that the effect of salicylic acid ($P \leq 0.05$) and NaCl ($P \leq 0.01$) on the root/stem ratio was significant (Table 1) but, interaction effect was no significant (Table 1). As compared to control (0 mM), with increasing of NaCl levels up to 50mM the root/stem ratio was increased. The

Table 1
Variance analysis of Salt and Salicylic acid levels on growth traits of in thyme (*Thymus vulgaris*)

		Mean of squares								
Source of variations	**df**	**Protein** (mg/mg FW)	**Peroxidase** (unit/mg protein)	**Catalase** (unit/mg protein)	**Soluble Sugar** (mg/g DW)	**Dry weight** (g)	**Leaf area** (cm^2)	**Fresh weight** (g)	**Root/ stem ratio**	**Height** (cm)
Saline	3	0.0004^{**}	0.003^{**}	57.56^{ns}	854.4^{**}	0.0001^{ns}	0.01^{**}	0.002^{**}	0.201^{**}	48.24^{**}
SA	3	0.00003^{ns}	0.002^{**}	320.6^{**}	55.95^{ns}	0.0001^{ns}	0.0002^{ns}	0.001^{ns}	0.088^{*}	0.687^{ns}
Saline*SA	9	0.0001^{*}	0.001^{**}	432.9^{**}	103.9^{ns}	0.0001^{ns}	0.0003^{**}	0.001^{**}	0.04^{ns}	12.59^{*}
Error	30	0.00003	0.00005	30.59	101.8	0.0001	0.0001	0.0005	0.027	5.318
% CV		14.05	12.1	12.35	16.73	22.99	10.28	17.11	16.76	11.19

Note: *, ** and ^{ns} are indicated significant at the level of 0.05, 0.01 and non-significant, respectively.

Table 2
Means comparison simple effects of salinity and salicylic acid on measured trait in Thyme (*Thymus vulgaris*)

Traits	**SA Levels** (ppm)				**Slat stress Levels** (mM)			
	0	150	300	450	0	50	100	150
Peroxidase (unit/mg protein)	0.033^{b}	0.073^{b}	0.044^{bc}	0.093^{a}	0.067^{b}	0.087^{a}	0.051^{c}	0.038^{d}
Catalase (unit/mg protein)	48.92^{c}	43.28^{c}	42.37^{c}	44.98^{a}	35.98^{b}	41.97^{b}	49.89^{a}	52.18^{a}
Root/ Stem ratio	0.9^{a}	0.89^{a}	1.00^{a}	1.17^{a}	0.980^{ab}	0.939^{b}	1.115^{a}	0.929^{b}
Fresh weight (g)	0.128^{b}	0.155^{a}	0.119^{b}	0.132^{a}	0.120^{b}	0.145^{a}	0.132^{ab}	0.137^{ab}
Dry weight (g)	0.049^{a}	0.056^{a}	0.050^{a}	0.053^{a}	0.047^{a}	0.054^{a}	0.053^{a}	0.054^{a}
Leaf area (cm^2)	0.130^{a}	0.116^{a}	0.095^{b}	0.62^{c}	0.099^{b}	0.108^{a}	0.100^{ab}	0.097^{b}
Protein content (mg/mg FW)	0.047^{a}	0.029^{b}	0.045^{a}	0.034^{b}	0.038^{ab}	0.035^{b}	0.041^{a}	0.041^{a}
Height (cm)	22.41^{b}	21.91^{a}	20.08^{a}	18.00^{a}	20.66^{a}	20.91^{a}	20.41^{a}	20.41^{a}
Soluble Sugar content (mg/g DW)	48.61^{a}	58.59^{a}	66.85^{a}	70.20^{a}	60.75^{a}	60.98^{a}	61.14^{a}	58.59^{a}

Note: Dissimilar letters is represents significant differences at probability level (P≤ %1)

root/shoot ratio was higher in 0 mM as compared to 50mM of NaCl but with increasing of NaCl levels up to 50mM was increased (Table2). In concentration of 300 mM of SA the root/stem ratio was increased. The highest of this trait was observed in application of 300 ppm SA×150mM NaCl (Table 3).

Leaf area

As shown in table 1, different levels of NaCl (P≤0.01) and interaction between NaCl × SA (P≤0.01) had a significant effect on leaf area but SA treatment had no significant effect (Table 1). Leaf area decreased with increasing of NaCl levels, significantly. Interaction between NaCl × SA treatment was showed that by SA application leaf area was increased under salt stress condition as the greatest of leaf area was observed in 150 ppm of SA (Table 3).

Fresh and dry weight

Different levels of NaCl and interaction between NaCl × SA had significant effect (P≤0.01) on fresh weight but had no significant effect on dry weight (Table 1). The highest and lowest of fresh weight was observed under 50 and 100 mM of NaCl levels, respectively (Table 2). Mean comparison of interaction between NaCl × SA was showed that SA was increased fresh weight as the highest fresh weight was obtained in 50 mM NaCl × 400 ppm SA (Table 3).

Soluble sugars

The effects of NaCl levels on soluble sugars was significant (P≤0.01), while no significant difference in application of SA and interaction between NaCl × SA (Table 1). With increasing of NaCl levels soluble sugars was increased, significantly, as the highest and the lowest content of soluble sugars were obtained in 150 and 0 mM of NaCl, respectively.

Protein content

Different levels of NaCl (P≤0.01) and interaction between NaCl × SA (P≤0.05) had a significant effect on protein content but SA treatment had no significant effect protein content (Table 1). The lowest content of protein was measured in 50 mM NaCl while with increasing of NaCl levels up to 50 mM, protein content was increased (Table 2). Interaction treatment of 150 mM NaCl × 300 ppm SA was increased protein content as compared to control (Table 3).

Quantitative activity of catalase (Q.A.C)

Different levels of NaCl had no significant effect on quantitative activity of catalase, while a significant effect was observed in application of SA and interaction treatment of NaCl × SA in significant level of 0.01 (Table 1). Mean comparison of interaction between NaCl × SA treatment was showed that quantitative activity of catalase was increased with increasing of SA levels. The highest of quantitative activity of catalase was obtained in 100 mM NaCl along with 150 ppm SA (Table 3).

Table 3
Interaction of salinity and salicylic acid on measured trait in thyme (*Thymus vulgaris*)

Salt levels	Salicylic acid levels	Peroxidase enzyme (unit/mg protein)	Protein (mg/mg FW)	Catalase enzyme (unit/mg protein)	Soluble sugar (mg/g DW)	Leaf area (cm^2)	Dry weight (g)	Fresh weight (g)	Root/ stem ratio	Height (cm)
0	0	0.028^{b}	0.05^{a}	32.94^{c}	38.48^{a}	0.146^{a}	0.046^{a}	0.116^{b}	1.02^{a}	19.66^{b}
	150	0.034^{ab}	0.034^{b}	58.3^{a}	48.83^{a}	0.133^{a}	0.056^{a}	0.154^{a}	0.77^{3}	24.33^{a}
	300	0.029^{b}	0.045^{ab}	52.87^{b}	54.74^{a}	0.116^{a}	0.053^{a}	0.146^{a}	0.93^{a}	23.66^{a}
	450	0.042^{a}	0.054^{ab}	51.59^{b}	49^{a}	0.126^{a}	0.04^{a}	0.096^{b}	0.87^{a}	22^{ab}
50	0	0.068^{b}	0.03^{a}	37.16^{c}	54.74^{a}	0.12^{a}	0.053^{a}	0.14^{a}	0.80^{a}	22.66^{a}
	150	0.118^{a}	0.033^{a}	20.62^{d}	62.08^{a}	0.116^{a}	0.056^{a}	0.175^{a}	0.80^{a}	24.33^{a}
	300	0.062^{b}	0.028^{a}	56.51^{b}	56.64^{a}	0.12^{a}	0.05^{a}	0.126^{a}	1^{a}	21.33^{a}
	450	0.044^{b}	0.027^{a}	61.1^{a}	60.88^{a}	0.11^{a}	0.066^{a}	0.186^{a}	0.95^{a}	19.33^{a}
100	0	0.036^{bc}	0.051^{a}	18.86^{c}	65.37^{a}	0.086^{b}	0.046^{a}	0.113^{ab}	1.03^{a}	21^{a}
	150	0.062^{ab}	0.042^{b}	65.72^{a}	71.77^{a}	0.103^{a}	0.05^{a}	0.116^{ab}	0.92^{a}	19^{a}
	300	0.064^{a}	0.045^{ab}	42.32^{b}	72.05^{a}	0.096^{ab}	0.046^{a}	0.106^{b}	1.12^{a}	19.66^{a}
	450	0.024^{c}	0.04^{b}	54.29^{ab}	58.23^{a}	0.096^{ab}	0.056^{a}	0.15^{a}	0.93^{a}	20.66^{a}
150	0	0.135^{a}	0.027^{b}	54.97^{a}	76.98^{a}	0.043^{c}	0.043^{a}	0.113^{a}	1.06^{a}	19.33^{a}
	150	0.112^{a}	0.034^{b}	35.13^{a}	61.78^{a}	0.08^{a}	0.053^{a}	0.146^{a}	1.25^{a}	16^{a}
	300	0.049^{b}	0.044^{a}	51.66^{a}	-	0.07^{ab}	0.063^{a}	0.15^{a}	1.41^{a}	17^{a}
	450	0.046^{b}	0.033^{b}	31.34^{a}	66.23^{a}	0.056^{bc}	0.053^{a}	0.12^{a}	0.96^{a}	19.66^{a}

Note: Dissimilar letters is represents significant differences at probability level (P≤ %1)

Quantitative activity of peroxidase (Q.A.P)

A significant difference was observed in NaCl treatment and interaction between NaCl × SA on quantitative activity of peroxidase (Table 1). The highest and the lowest of quantitative activity of peroxidase were measured in 150 and 0 mM NaCl levels, respectively (Table 2). Mean comparison of interaction effects was showed that the highest of quantitative activity of peroxidase was obtained in 50 mM NaCl × 150 ppm SA (Table 3).

Discussion

Water deficit and salt-specific or ion-excess effects are two outcomes of Salinity, which inhibit plant growth (Munns et al., 2006). Salinity has been shown to reduce the imbibition of water by roots because of lowered osmotic potentials of the substrate, and to cause changes in metabolic activities leading to the reduction in plant growth (Meneguzzo *et al*., 1999; Jaleel *et al*., 2007). In this study, plant height was decreased under salinity condition The most important plant response to salinity is reduction in growth as with increasing salinity above the tolerance threshold, the plant growth is reduced and the height is decreased (Homaei, 2002).

In our experiment, although the increasing trend of fresh and dry weight was not observed with increasing of salt stress levels, but these traits was higher in the most of salinity treatments as compared to the control (0 mM NaCl). It seems that the increase in fresh and dry weight under salinity condition may be due to increasing of ion uptake and subsequent absorption of water, which finally resulted to increasing of cell turgor pressure and plant growth. Teimouri and Jafari (2007) was reported that, fresh and dry weight of shoot and root and stem number in Alfalfa (*Medicago sativa*), was increased in respond to salinity stress and demonstrated that these responses are indirect and physiological effects related to stress tolerance.

Root to stem ratio (Root/stem ratio) increased with salt stress, having been more visible in higher concentrations of NaCl. It has been reported that shoot growth is more sensitive to salinity than root growth (Da Silva *et al*., 2008). This sensitivity may be explained due to an imbalance among cations as a result of the complex interaction in the xylem transport system (Munns, 1993). This was caused by both a continuous decline of the shoot biomass and a sharp increase of the root dry weight, the latter

being possibly associated to a reallocation of Photosynthesis into the roots. High root/stem ratio can improve the salt tolerance of plants. (Lalkhajanchi *et al*., 2007). Increased Root to stem ratio may resulted to increased water uptake, which subsequently reduced water consumption (Greenway and Munns, 1980).

Leaf area reduced as salt concentration increased. According to Da Silva *et al*. (2008), leaf area is the most sensitive growth parameter in response to high salt levels. SA application under salt stress resulted in considerable increases in leaf area. The application of salicylic acid, acetylsalicylic acid or other analogues of SA, to leaves of corn and soybean accelerated their leaf area and dry mass production, but plant height and root length remained unaffected (Khan *et al*., 2003). This positive effect of SA could be attributed to an increased Carbon dioxide (CO2) assimilation and photosynthetic rate and increased mineral uptake by the stressed plant under SA treatment. The promoting effect of SA on the leaf area was attributed to its important roles on activating cell division and the biosynthesis of organic foods. The enhanced activities of the photosynthetic enzymes in SA-treated plants might have ultimately resulted in an improved photosynthetic rate and plant biomass in both stressed and unstressed conditions (Aftab *et al.*, 2011).

In this study, soluble sugars accumulation was increased with increasing of salt stress levels. Our results are in agreement with those from the studies by Hoseini (2010) in *T. vulgaris*, Najafi *et al*. (2010) in *Satureja hotensis* and Abd El-Wahab (2006) in *Foeniculum vulgare*. Soluble carbohydrates and free amino acids have been mentioned as important compounds in osmoregulation in plants underwater and salt stresses (Hare *et al*., 1998). Accumulation of these compatible solutes reduces osmotic potential in the cytoplasm and contributes to maintaining water homeostasis among several cellular compartments, playing a leading role in osmoprotection, osmotic adjustment, carbon storage, and radical scavenging, despite a significant decrease in net CO_2 assimilation (Sairan and Tyagi, 2004).

According to our results, maximum and minimum of protein content was obtained in 0 and 50 mM NaCl, but with increasing of salt levels up to 50 mM NaCl, protein content was increased. Proteins that accumulate in plants under saline conditions may provide a storage form of nitrogen that is re-utilized later (Parvaiz and Satyawati, 2008) and may play a role in osmotic adjustment. In this research, protein synthesis in higher concentrations of NaCl treatments can be considered as a mechanism for osmotic adjustment and more adaptation to salinity stress (Zahra *et al*., 2010).

There was a higher level of peroxidase activity in salt stressed plants, Maximum of peroxidase activity in salt-treated plants was obtained at 150 mM NaCl. To overcome oxidative stress, plants have developed enzymatic and non-enzymatic antioxidant defense mechanisms to scavenge ROS (Ghasemzadeh and Jaafar, 2013).

The results of this study showed different levels of salinity had no significant effect on quantitative activity of catalase but Observed positive correlations among peroxidase enzyme activities, Soluble Sugar and protein content suggest that increased defense mechanism against the reducing negative effects salinity stress.

Interestingly, SA application enhanced the activities of the antioxidant enzymes CAT and POX in salt stressed plants. These results are in agreement with the observations made by (Aftab *et al*., 2011) on (*Artemisia annua* L.), who advocated that relatively high levels of peroxidase and superoxide dismutases were produced in response to SA application under salinity stress. Under stress conditions such as salinity and drought, plants produce reactive oxygen species, such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, which are harmful to plant growth and productivity (Mishra and Choudhuri, 1999). Thus, to maintain metabolic functions under stress, scavenging of reactive oxygen species is essential. In this regard, catalase, peroxidase and superoxide dismutases appear to play an essential protective role in scavenging ROS (Jaleel *et al*., 2009). SA-induced catalase, peroxidase and superoxide dismutases activities in salt treated *Thymus vulgaris* plants indicates that SA can play a critical role in modulating the cell redox balance, thereby protecting plants against oxidative damage (Yang *et al*., 2004). This

increased antioxidant enzyme activity might be due to SA's regulatory role at transcriptional and/or translational levels (Ghasemzadeh and Jaafar, 2013).

Our results indicate that SA application after exposure to salinity stress was improved Stem High, fresh weight, leaf area, protein content, and quantitative activity of catalase and peroxidase enzyme. Similar results were reported by other researchers on different crops (Borsani *et al*., 2001; Gunes *et al*., 2007; Elwana *et al*., 2009; Karlidag *et al*., 2009; Najafian *et al*., 2009). Increasing of growth traits in salt effected plants in response to SA might be related to the protective role of SA on membranes that might increase the tolerance of plants to salt stress (Aftab *et al*., 2010). Also, SA application under salinity condition may be activated SA-induced antioxidant function and metabolic activity in plants and resulted to SA-mediated increase in dry matter (Gunes *et al*., 2007). Although, The effect of salicylic acid on plant physiological processes varies depending on species, developmental stage, SA concentration and environmental conditions (Ghasemzadeh and Jaafar, 2013).

Based on the results of this study, physiological and morphological characteristics of Thymus vulgaris were affected by salinity stress. On the other side, foliar application of SA was improved salinity tolerance through increasing in activity of antioxidant enzymes and growth traits it therefore appears that SA can generally be used as a growth regulator to enhance plant growth and yield.

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