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The influence of traumatic acid on the growth and metabolite content of the green alga *Chlorella vulgaris* Beijerinck

Anna Pietryczuk[1], Alicja Piotrowska, Romuald Czerpak

University of Białystok, Institute of Biology
ul. Świerkowa 20B, 15-950 Białystok, Poland

Key Words: traumatic acid, chlorophyll *a* and *b*, total carotenoids, monosaccharides, water – soluble proteins.

Abstract

The effect of exogenous traumatic acid, (2E)-dodecene-1,12-dioic acid (TA), at concentrations of 10^{-6} - 10^{-3} M on the growth and development of the unicellular green alga *Chlorella vulgaris* was examined over 7 days. TA plays an important role in algal growth and development. Treatment with 10^{-5} M TA resulted in the greatest increase in cell numbers, by 77%, chlorophyll *a* content, by 126%, chlorophyll *b,* by 45%, total carotenoids, by 65%, monosaccharides, by 111%, and water-soluble proteins, by 37%, in relation to the control. After three days of treatment with 10^{-5} M TA cultures were analysed by SDS-PAGE, which showed the presence of new polypeptides with molecular weights 13-103 kDa.

[1] Corresponding author e-mail: *annapiet@uwb.edu.pl*

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INTRODUCTION

Plants in their natural environment are exposed to various biotic and abiotic stresses. In response to wounding, herbivores and pathogen attacks, polyunsaturated fatty acids level increase in plants tissues. The fatty acids linoleic and linolenic acid are released from cell membrane lipids by wound–inducible phospholipases (León et al. 2001). Linoleic and linolenic acids are transformed in reactions catalysed by lipoxygenases (LOXs) into 13-hydroperoxy linoleic acid (13-HPOD) and 13-hydroperoxy linolenic acid (13-HPOT), respectively (Grechkin 1998, Grechkin et al. 2000). In the second step of this process the hydroperoxy fatty acids are converted to jasmonic acid (JA) by the action of allene oxide synthase (AOS) or to traumatic acid ((2E)-dodecene-1,12-dioic acid, TA), by the action of hydroperoxide lyase (HPL) (Sivasankar et al. 2000; Grechkin 2002; Howe, Schilmiller 2002).

TA is the product of traumatin ((10E)-12-oxo-dodecenoic acid) non-enzymatic autooxidation. Traumatin commonly occurs in the leaves, fruits and seeds of many plants species and is considered to be a natural wound hormone. Traumatin is synthesised by young plants and is increasingly observed in the esterified form with increasing age of the plant (Zimmerman, Coudron 1979). 10-oxo-(8E)-decenoic acid (ODA), with similar properties as a plant traumatin, has been identified in fungi (Mau et al. 1992; Champavier et al. 2000).

TA was first isolated from *Phaseolus vulgaris* L. It acts as a growth stimulator and induces cell proliferation and elongation, although not as strongly as has been observed by traumatin. Stimulation of growth of vascular plants and fungi has been reported using traumatin and ODA (Strong, Kruitwagen 1967; Zimmerman, Coudron 1979; Mau, Beelman 1996, Grechkin 2002). However, it is also been proposed that traumatin causes DNA and protein damage, and possesses cytotoxic properties (Grechkin 2002). ODA, at high concentrations (500 μM and 1 mM), has also been shown to exhibit cytotoxic properties and inhibit the growth of *Agaricus bisporus* mycelium (Champavier et al. 2000).

The roles of JA and other stress hormones in plant growth and metabolism are known to a considerable degree (Bostock 1999; León, Sanchez-Serrano 1999; Christov et al. 2001; Westernack et al. 2006). However, data concerning the biological activity of TA are still limited. For this reason, we examined the influence of TA, in concentrations from 10^{-6} to 10^{-3} M, on changes to the growth and essential metabolite content in *Chlorella vulgaris* cells. *C. vulgaris* (Chlorophyceae) is used as a model system because it can be cultivated in simple media and is characterised by rapid cell division. Moreover, variations in signalling molecules and biochemical responses can be perceived in the same cell.

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MATERIALS AND METHODS

Plant material and growth conditions

Axenic cultures of the green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae) came from the collection of the Department of Plant Biochemistry at the University of Białystok. *Chlorella vulgaris* cells were cultivated for 7 days under controlled conditions at 25 ±0.5°C. Illumination was supplied during a 16-h photoperiod (8-h dark period) by a bank of fluorescent lights yielding a photon flux density of 50 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at the surface of the tubes. PAR was measured with a phytophotometre FF-01 (SOMOPAN, Poland). Permanent synchronous growth was established according to the method of Pirson and Lorenzen (1966). The culture medium used was modified Knop's medium with the following components: 0.5 g KNO_3, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 0.003 g H_3BO_3, 0.002 g $MnCl_2 \cdot 4H_2O$, 0.0003 g NH_4VO_3, 0.0002 g $ZnSO_4 \cdot 7H_2O$, 0.0001 g $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ in 1 l of distilled water. The culture medium and glassware were sterilized by autoclaving at 125°C for 25 min. The pH of the medium was adjusted to 6.8 with 1 M NaOH. *C. vulgaris* were cultured in Erlenmeyer flasks (500 ml) containing 250 ml of medium and shaken at 150 rpm in a rotary shaker. TA was obtained from Sigma Chemical Co. (USA). TA dissolved in 0.1 M NaOH was applied at four concentrations: (a) 10^{-3} M, (b) 10^{-4} M, (c) 10^{-5} M and (d) 10^{-6} M. An equal amount of 0.1 M NaOH was added to the control. Cultures were conducted in four replicates. Algal samples for determination of the number of cells and the content of some metabolites were collected on the 1st, 3rd, 5th and 7th days of cultivation. The culture, from which the inoculum was taken, was in logarithmic growth phase. The initial cell density was about 10.6×10^5 cells per ml in all flasks.

Cell number determination

The number of *C. vulgaris* cells was determined by direct counts of cells in the growth medium using a Bürker chamber.

Monosaccharide determination

In order to assess monosaccharide concentrations per unit fresh weight of the *C. vulgaris,* 10 ml subsamples of the algal culture were collected by centrifugation. The monosaccharides were extracted in ethanol for 24h, according to the Somogyi method (1954), and the absorbance measured with a Shimadzu UV-Vis 1201 spectrophotometer.

Determination of photosynthetic pigments

The content of photosynthetic pigments followed homogenization of fresh *C. vulgaris* in 99.9% methanol at 70°C for 30 min. (Wellburn 1994). The absorbance of the extract was measured with a Shimadzu UV-Vis 1201 spectrophotometer at 652.4 and 665.2 nm for chlorophylls *a* and *b* and at 470.0 nm for carotenoids. The amounts of photosynthetic pigments present in the methanol extract were calculated according to equations in Wellburn (1994).

Determination of water -soluble proteins

The water soluble protein content was calculated following homogenization of *C. vulgaris* biomass and extraction overnight in 0.1 M NaOH at 4°C. The concentration of proteins was determined spectrophotometrically as per Lowry et al. (1951), using Folin phenol reagent with a protein kit calibrated with bovine serum albumin as the standard. The absorbance of the extracts was measured with a Shimadzu UV-Vis 1201 spectrophotometer.

SDS-PAGE

Soluble proteins for SDS-PAGE were extracted from three-day-old algal cultures. The cell suspension was passed through a Whatman filter 1 under pressure and quickly homogenised using liquid nitrogen and subsequently a lysis buffer containing 0.5 M Tris-HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue. The cell extract was incubated at 100°C for 5 min. then centrifuged at 800×*g* for 10 min. to pellet unbroken cells and cell debris. The supernatant was removed and cenrifugated at 10,000×*g* for 5 min. 20 μl samples of SDS: protein, at a ratio of 4:1 (v/v), were loaded onto a 12% polyacrylamide gel containing 0.1% SDS and the buffer system as per Laemmli (1970). Gels were run at 20°C at a constant current of 15 mA for approximately 4 h, and then stained with Coomassie brilliant blue (Fairbanks et al. 1971).

Replication and statistical analysis

Each treatment consisted of 4 replicates and each experiment was carried out on at least two different occasions. A minitab statistical package was used to carry out a one-way ANOVA. The Student's t-test was used to estimate the difference between means at a 5% level of significance.

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RESULTS

Changes in *C. vulgaris* cell numbers and levels of certain essential metabolites were detected during the seven day incubation following addition of TA at concentrations of 10^{-6} - 10^{-3} M.

The highest increase in algal cell numbers, by 77% compared to the control, was observed on the 3rd day of cultivation in cultures that had 10^{-5} M TA added (Fig. 1). All of the incubations with TA additions showed a decrease in cell numbers after the third day. By the seventh day of the experiment the incubations with 10^{-3} M TA and 10^{-6} M TA additions showed reductions in cell numbers of 13% and 10% respectively, in relation to the control.

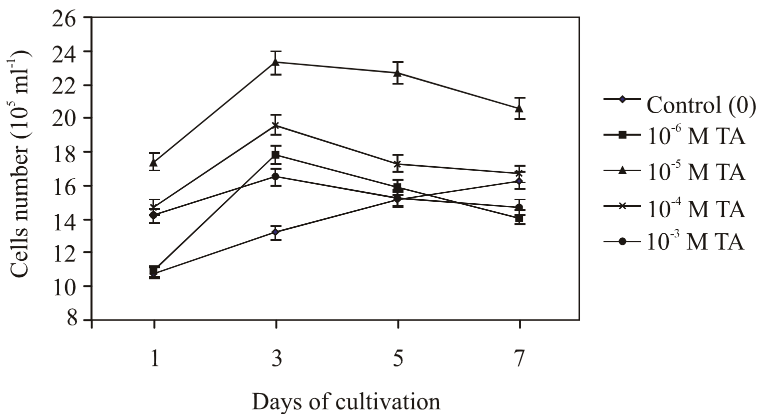

Fig. 1. The effect of various concentrations of traumatic acid on *Chlorella vulgaris* cell numbers during a 7-day incubation (SE<5%).

The addition of TA caused at least a twofold increase of monosaccharide accumulation in *C. vulgaris* cells (Fig. 2). The addition of TA at a concentration of 10^{-5} M resulted in an increase of monosaccharide content by 111% compared to the control. The 10^{-6} M TA addition caused the weakest stimulatory effect on monosaccharide levels, with an increase of only 27% by the 3rd day of cultivation. The monosaccharide contents of cells in all incubations with TA additions fell, compared to the control, after day three of the incubation.

The results of TA additions on chlorophyll *a* and *b* concentrations in *C. vulgaris* are presented in Fig. 3 and 4. TA additions increased chlorophyll *a* accumulation throughout the 7-day cultivation in all samples, compared to the controls. The greatest increase was seen in the 10^{-5} M TA treated samples on the third day of the experiment. In contrast, TA additions exhibited weaker

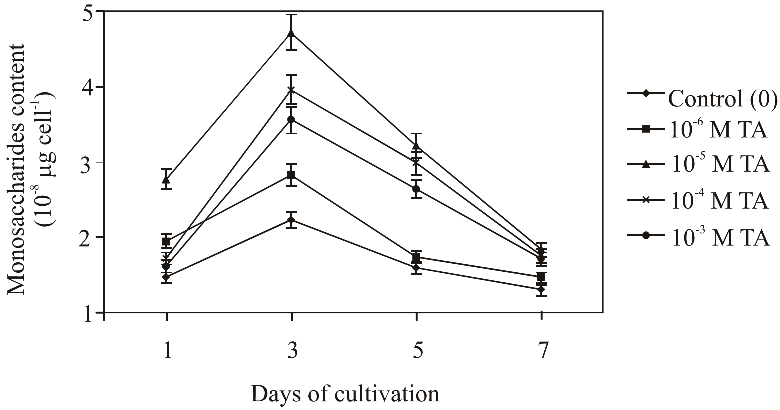

Fig. 2. The effect of various concentrations of traumatic acid on the monosaccharide content of *Chlorella vulgaris* during a 7-day incubation (SE<5%).

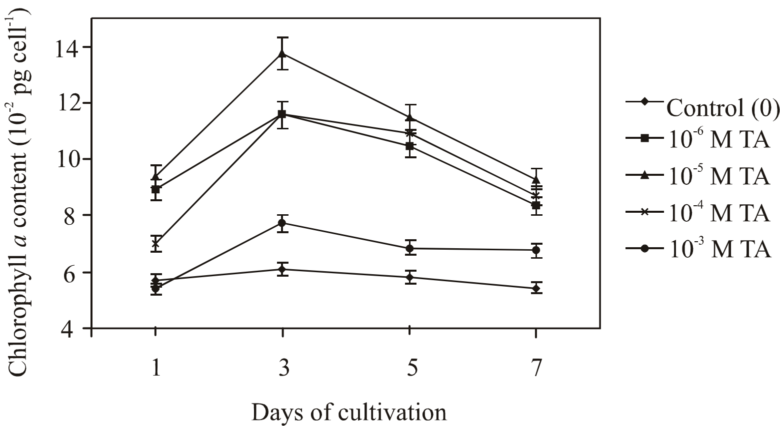

Fig. 3. The effect of various concentrations of traumatic acid on the chlorophyll *a* content of *Chlorella vulgaris* during a 7-day incubation (SE<5%).

stimulation on chlorophyll *b* accumulation in *C. vulgaris* cells. The greatest increase in chlorophyll *b* concentrations, up by 45% compared to the control, was seen in the 10^{-5} M TA-treated algae on the third day of incubation. Additions of 10^{-3} M TA were characterised by a weak stimulatory effect on the chlorophyll *a* content and acted as an inhibitor of the chlorophyll *b* accumulation in *C. vulgaris* cells.

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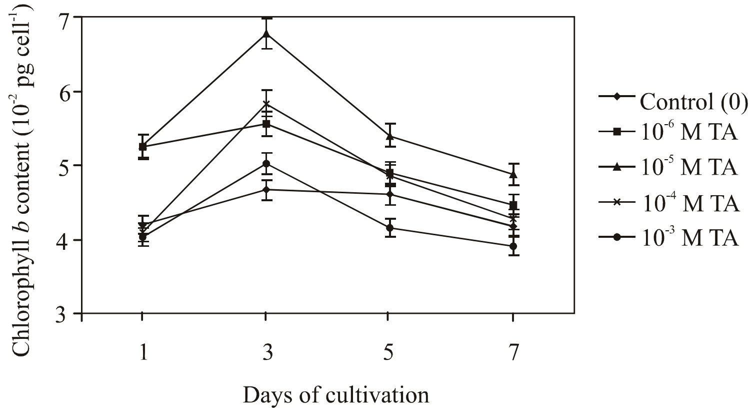

Fig. 4. The effect of various concentrations of traumatic acid on the chlorophyll *b* content of *Chlorella vulgaris* during a 7-day incubation (SE<5%).

Changes in the carotenoid contents of *C. vulgaris* cells caused by TA additions are presented in Fig. 5. TA additions at a concentration of 10^{-5} M resulted in an increase of total carotenoid contents by 48-65%, compared to control cultures. Carotenoid accumulations in the cells grown in the presence of the highest concentrations of TA (10^{-3} M) were inhibited by 10-24%, compared to the control.

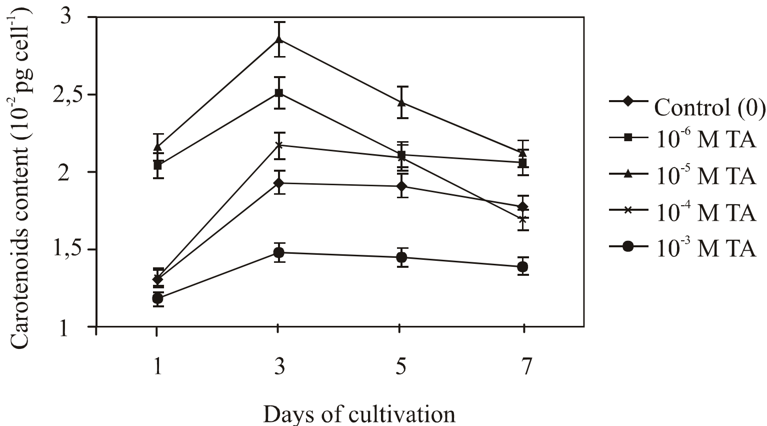

Fig. 5. The effect of various concentrations of traumatic acid on the carotenoid content of *Chlorella vulgaris* during a 7-day incubation (SE<5%).

Changes in water-soluble protein contents in *C. vulgaris* cells resulting from the various TA additions are presented in Fig. 6. The greatest increase in the water-soluble protein levels, by 33-37% compared to the control, occurred in

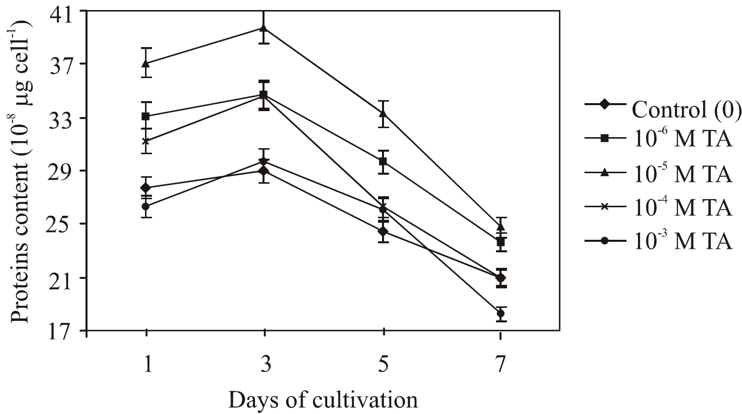

Fig. 6. The effect of various concentrations of traumatic acid on the content of water-soluble proteins in *Chlorella vulgaris* during a 7-day incubation (SE<5%).

the incubations with additions of 10^{-5} M TA. A slight increase of protein accumulation was observed in the cells cultivated in the presence of 10^{-3} M TA between the third and fifth days of the experiment. The SDS - PAGE analysis of total cellular proteins isolated from the *C. vulgaris* show that under the influence of the most stimulating concentration of TA (10^{-5} M), about 13 new proteins, with a range of molecular weights from 13-103 kDa, were detected that were not observed in the control cultures (Fig. 7). Further, 5 polypeptide bands with molecular masses of 85, 75, 30, 26 and 15 kDa appeared to be in higher concentrations under the influence of TA additions than in the controls. In control cultures 8 proteins, with molecular weights of 18, 21, 32, 39, 56, 76, 85 and 100 kDa, were detected (Fig.7, Tab. 1).

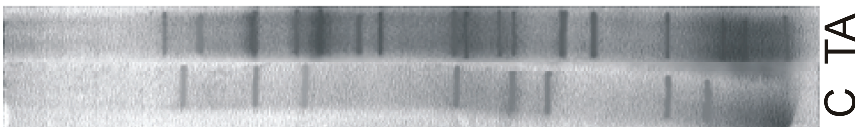

Fig. 7. SDS-PAGE of the total water-soluble proteins isolated from *Chlorella vulgaris* cells cultivated in the presence of 10^{-5} M traumatic acid (TA) compared to the control culture (C).

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Table 1

The molecular weights of proteins isolated from *Chlorella vulgaris* cells cultivated in the presence of traumatic acid and control group. The molecular weights of proteins that accumulated the highest amounts in the cells are shown in bold.

Molecular wight of proteins (kDa)	
Control	TA
100	103
85	90
76	**85**
56	77
39	**75**
32	68
21	66
18	56
	49
	42
	39
	30
	26
	21
	17
	15
	13

DISCUSSION

It is known that TA plays an active role in the different plant responses to environmental stresses, especially mechanical wounding and pathogen attacks. However, there is little information regarding the affect of TA on growth and metabolism of lower plants, e.g. green algae. Our research shows that under the influence of TA additions cell numbers and metabolite contents of *C. vulgaris* alter during a 7-day incubation.

Additions of 10^{-5} M TA resulted in almost double the number of *C. vulgaris* cells compared to control incubations. Previous data on vascular plants show that traumatin and TA cause intensive proliferation and elongation of the parenchymous lining cells of bean pods (*Phaseolus vulgaris* L.), especially in young plants (Zimmerman, Coudron 1979). The greatest increase in algal cell numbers was observed during the first three days of the experiment, after which (between the fifth and seventh days of incubation) the activity of TA began decreasing. This probably results from TA degradation, as has been reported in older beans (Zimmerman, Coudron 1979) where the free TA is converted to its esterified, biologically inactive form. Moreover, TA causes quick cell divisions resulting in overgrowth, defined as tumours (Treshow 1955).

The results presented here show that TA incites not only increased cell division but also causes changes in cellular levels of essential metabolites in *C. vulgaris*. A 10^{-5} M TA addition caused a significant increase in accumulation of chlorophylls and total carotenoids. An increase in the biosynthesis rate and accumulation of pigments occurs in plants cells under the influence of various biotic and abiotic stresses. These pigments have protection functions against UV irradiation and oxidative stress (Vasil'eva 2003; Glaeser, Klug 2005; Bashan et al. 2006). A rapid increase in the content of chlorophylls and total carotenoids in algal cells was also observed following treatment with JA (Czerpak et al. 2006). Moreover, Saniewski et al. (1998 a, b) showed that JA induces accumulation of anthocyanins in peach shoots and tulip bulbs. It can be supposed that TA acts in the same way in plants.

A plant tissue response to wounding or pathogen attack has been observed to be the accumulation of soluble sugars, especially glucose, fructose and sucrose (Gillet al. 2003). A twofold increase in monosaccharide content was detected in *C. vulgaris* cells treated with exogenous TA. Soluble sugars are important signalling molecules, involved in the signal transduction pathway leading to modifications in expression of defence genes. It can be supposed that TA also induces sucrose hydrolysis and gluconeogenesis. With the addition of JA a rapid increase in monosaccharide concentration was reported in vascular plants and algal cells (Bogatek et al. 2002, Czerpak et al. 2006).

In response to the action of the stress phytohormones, such as JA, abscisic acid (ABA) and the commonly occurring phenolic acid, salicylic acid (SA), in plant tissues quantitative changes in protein patterns have been observed. JA induces a protein with molecular weight 96 kDa in vascular plants (Tarchevsky et al. 2001). Considerable changes in protein content have also been shown in the green algae *Scenedesmus incrassulatus* and *C. vulgaris* following treatment with jasmonates (Christov et al. 2001, Czerpak et al. 2006). The observed increase in content of water-soluble proteins, and the appearance of new polypeptides, in *C. vulgaris* cultivated in the presence of the optimal concentrations of TA is probably connected with the major role of TA in plants as a stress agent. Sivasankar et al. (2000) proposed a model for the activation of defence genes in the tomato (*Lycoperiscon esculentum*). According to that model, traumatin induces prosystemin synthesis, which is then converted into systemin, an 18-aminoacid peptide. The systemin is dislocated from the wounding place to the rest of the plant. The new proteins identified by the SDS-PAGE in the *C. vulgaris* treated with TA could be defence proteins produced in response to the hormone.

The influence of oxylipins on the growth of bacterial and fungal plant pathogens was investigated in order to better recognize their biological activities. Most analysed derivatives of fatty acids inhibit the growth of such

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pathogenic bacteria and fungi. A 6C aldehyde by-product of the traumatin biosynthesis pathway, (2E)-hexenal, demonstrated highly antibacterial properties, reducing the growth of bacteria even at a concentration of 10 μM. Moreover, the bacteria exhibited sensitivity to 13-hydroperoxy linoleic acid (13-HPOD). Antibacterial activities were not detected in response to treatment with TA, although TA reduced the growth of pathogenic fungi. Antifungal properties have been shown in response to the 13-HPL products, 13-HPOD and 13-HPOT (Prost et al. 2005). It is supposed that the mechanisms by which the oxylipins, including TA and C6 aldehydes, influence defence gene expression and reduce growth of the plant pathogens are closely related.

The molecular mechanism of TA action is still unknown. Asafova et al. (2005) reported that TA enhances the tyrosine phosphorylation of pea proteins with molecular weights of 19, 20, 22, 26, 31, 42 and 74 kDa. However, the tyrosine phosphorylation of proteins with molecular weights 36, 47 and 49 kDa was inhibited under the influence of TA. It is probable that TA binding to the potential receptor induces the kinases activity and phosphorylation of the proteins which initiates a further signalling cascade.

TA, and similarly JA, ABA and SA, are important signalling substances in response to various stresses. *C. vulgaris* incubated with exogenous TA is characterised by increased accumulation of monosaccharides, photosynthetic pigments and water-soluble proteins, compared to controls. Moreover, the presence of TA induces proteins in the alga that are not detected in control cultures. Further research and understanding of the biological roles of TA in lower plants may also aid the comprehension of the molecular mechanisms of TA action in vascular plants.

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