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Effects of Acetylsalicylic Acid on Germination, Growth and Chlorophyll Amounts of Cucumber (*Cucumis sativus* L.) Seeds

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Abstract: Germination activities, various growth parameters (primary root length, hypocotyl length, primary leaf length, plant length and increase in fresh weight) and chlorophyll (a+b) amounts of cucumber seeds exposed to 0, 10^{-5}, 10^{-4}, 10^{-3} and 0.5×10^{-2} M aqueous solutions of acetylsalicylic acid (ASA) for 48 h were established. While 0.5×10^{-2} M ASA significantly prevented germination activity of the seeds, other concentrations did not produce any effect, either positive or negative. Meanwhile, 0.5×10^{-2} M ASA inhibited radicle growth of the germinated seeds, while 10^{-5} M ASA increased radicle growth. Other concentrations of ASA did not affect radicle growth. The following findings were obtained from the one-week seedlings exposed to ASA for 48 h: 0.5×10^{-2} M ASA prevented growth (lengthening) of root, hypocotyl, leaf and plant and increased chlorophyll (a+b) amount with an increase in fresh weight. Contrary to 0.5×10^{-2} M ASA application, these features of the seedlings (except for the leaf length) were encouraged by 10^{-5} M ASA. 10^{-3} M ASA only prevented root growth and reduced chlorophyll (a+b) amount. Other concentrations of ASA did not bring about any positive or negative effect on the features studied.

Key words: Acetylsalicylic acid, cucumber, germination, growth, chlorophyll

INTRODUCTION

During their metabolic activities, plants synthesize a multitude of secondary metabolites that are not necessary for their primary metabolisms. At present, it is known that plants use these metabolites in a variety of activities like defense, protection, adaptation to the environment, survival and reproduction. Phenolic compounds constitute an important class of plants' secondary metabolites. One of these compounds is Salicylic Acid (SA), which is an aromatic organic acid. Commercial manufactured form of salicylic acid is Acetyl Salicylic Acid (ASA). In aqueous solutions, acetylsalicylic acid hydrolyzes completely to its agent SA (Mitchell and Broadhead, 1967).

Salicylic acid dose-dependently plays important roles in the growth and development processes of plants. These include exercising a thermogenic effect (Raskin *et al*., 1989), stimulating adventive root development (Kling and Meyer, 1983), showing a herbicidal effect (Shettel and Blake, 1983), reducing leaf shedding (Ferrarese *et al*., 1996), providing resistance against pathogens (Salisbury and Ross, 1992), inhibiting ethylene biosynthesis (Carswel *et al*., 1989), modifying the quality and quantity of proteins (Jung *et al*., 1993; Mersie and Singh, 1993; Çanakci, 2003) and providing endurance against stress (Ágnes *et al*., 2005; Rajasekaran *et al*., 2001; Adalberto *et al*., 2002; Senaratna *et al*., 2000). Such examples and many other phenomena caused by SA suggested to some researchers that this substance could be another plant growth regulator (Raskin *et al*., 1989; Raskin, 1995; Losanka *et al*., 1997; Rajasekaran and Blake, 1999).

The literature about the effects of SA and ASA on germination in plants is limited. It was reported in a study conducted on varieties of tomatoes (*Lycopersicon esculentum* L.) using different concentrations of various phenolic compounds (benzoic acid, chlorogenic acid, ferulic acid, p-hydroxybenzoic acid, salicylic acid and 2,4-diacetyl phloroglucinol) that strong phytotoxic effects were observed in 10 μM concentration of chlorogenic acid, in particular. It was stated in the same study that high concentrations of phenolic compounds (100 and 1000 μM) prevented germination, reduced root and shoot fresh weight, while lower concentrations did not produce a significant difference in comparison to controls, but brought about different effects when compared to one another. Only 1 μM concentration of 2,4-diacetyl phloroglucinol (phl) increased fresh weight significantly, relative to the control (Jung *et al*., 2001). In a study exploring the effects of short- and long-term SA exposure on germination of cabbage (*Brassica oleracea* L.), tomato (*Lycopersicon esculentum* L.) and cucumber (*Cucumis sativus* L.) seeds, it was seen that long-term applications were more effective than short-term applications in preventing germination (K'Opondo *et al*., 2001). It was reported that high concentrations of ferulic acid had a

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higher inhibitive effect, than lower concentrations, on germination of soy bean (*Glycine max* L. Merill) seeds and led to formation of dwarf roots with necrotic appearance (Colpas *et al*., 2003). It was established that high concentrations of various phenolic compounds, prevented seed germination of *Atriplex triangularis*. Inhibition of germination by exogenous applications of all highly active phenols (10^{-2} M) except salicylic acid was alleviated by the application of gibberellic acid and kinetin (Khan and Ungar, 1986). Coumarin application was seen to prevent germination in *Anthoxanhtum odoratum*, particularly in high concentrations and encouraged root formation in lower concentrations (Yamamoto and Fujii, 1997). It was established that high concentrations of various phenolic compounds (ferulic, gallic, p-hydroxybenzoic acid and p-vanillin) prevented seed germination of six different types of wild herbs. Lower concentrations of these compounds, on the other hand, were seen either to remain ineffective or to show a stimulator effect (Regiosa *et al*., 2004).

There is no sufficient information available as to how ASA influences weight change in plants or what effects it shows on their pigment and protein contents depending on concentrations. It was reported in a study by Shettel and Blake (1983) that SA and p-hydroxybenzoic acid application prevented growth of seedling and reduced dry weight increase in corn (*Zea mays* L.), soy bean (*Glycine max* L.), oat (*Avena sativa* L.) and three wild plants. It was observed in barley plants germinated and grown in different salicylic acid concentrations that secondary leaf area increase and root development were prevented, while protein and chlorophyll (a+b) amount decreased parallel to concentration increase (Pancheva *et al*., 1996). High concentrations of ASA were reported to inhibit root and coleoptile growth (Larque-Saavedra, 1978). In a study carried out with disks taken from primary leaves of one-week bean seedlings, chlorophyll a and b amount decreased parallel to the increase in ASA concentration, while carotenoid amount remained unchanged, but fresh weight loss and protein destruction increased (Çanakci, 2003). The fact that the effects of salicylic acid on plants vary depending on the type of plant, life period during application, concentration applied, manner and duration of application makes it difficult to explain the physiological effects of SA. This situation can be clarified only through a high number of research studies. As it is known, seed germination is a complicated process which aims at the mobilization of reserve substances and thereby includes various resulting hormonal modifications, gene induction and a high number of enzyme syntheses. We think that it is important to know whether SA, a new plant hormone, has any effect on this process which starts with swelling. Therefore, in this study, effects of different concentrations of ASA on germination, various growth parameters and chlorophyll (a+b) amount of cucumber (*Cucumis sativus* L. Beit Alpha) were examined.

MATERIALS AND METHODS

Preparation of plant materials and acetylsalicylic acid solutions: 2006 producea, certified (H007) cucumber seeds (*Cucumis sativus* L. Beit Alpha) were used as the plant material, 99.5% pure crystal acetylsalicylic acid (Sigma) and 1 mL 95% ethanol (Merk) to dissolve crystal ASA were used as the chemicals and 0.05 M NaOH, 0.05 M HCl and de-ionized water were used for pH adjustments.

Firstly, 0.5×10^{-2} M stock solution of ASA was prepared. For this purpose, crystal ASA was dissolved in ethanol (Shettel and Blake, 1983) and de-ionized water was used. Then water was diluted from this stock solution with de-ionized water and solutions of ASA in concentrations of (0, 10^{-5}, 10^{-4}, 10^{-3} and 0.5×10^{-2} M) were prepared. pH of all solutions were adjusted to 4.5 to 5 (Larque-Saadvera, 1978). pH measurements were conducted in JENWAY 3040 ion analysis and pH meter apparatus. Sartorius BL brand precision scales were used in weight measurements.

Establishment of germination activity and radicle growth rate: In order to determine the germination rate of seeds, 100 seeds were used for each ASA concentration. Cucumber seeds were left to swell in 100 mL ASA solutions in dark and at 25°C for 5 h. Then these seeds were sowed on two-layered filter papers moistened with swelling environment liquid and laid in Petri boxes of 11 cm diameter. After the seeds were sowed, all boxes were put into a drying oven adjusted to 25°C and left to wait for 48 h starting from the time of moistening. At the end of this period, seeds of each group were thoroughly washed with tap water. The washed seeds were laid down on two-layered filter papers moistened with tap water in sterile Petri boxes of 11 cm diameter and put into a dark drying oven adjusted to 25°C. The number of germinated seeds (principle: emergence of radicle from the seed cover) was established at 12 h intervals for 3 days starting from the time of moistening. Germination rate was expressed as % for each group. Additionally, the radicle length of germinated seeds in all groups was measured using a millimetric ruler. For this purpose, 20 germinated seeds were used for each group.

Establishment of growth parameters: In order to determine growth parameters, from the germinated seeds to which ASA was applied for 48 h after the moistening

time and which were thoroughly washed with tap water, as described above, those with homogeneous radicle length (on the basis of the most common root length in each group) were selected. Germinated seeds from all groups were transplanted with a certain arrangement between the folds of filter paper moistened with 100 mL muddy tap water in 250 mL vials each. Then the vials were put into growth cabinets (light intensity: 350 μmol $m^{-2}s^{-1}$ (natural light), temperature: 25±2, photoperiod: 12 h). The seedlings were kept in these growth cabinets till they were one-week after the time of moistening. Primary root length, hypocotyl length, leaf length and plant length were determined. For this purpose 20 germinated seeds were used for each group.

Determination of fresh weight: In order to determine fresh weight, from the germinated seeds to which ASA was applied for 48 h after the moistening time and which were thoroughly washed with tap water, as described above, those with homogeneous radicle length (on the basis of the most common root length in each group) were selected and their first weights were individually determined. Then, germinated seeds from all groups were transplanted with a certain arrangement between the folds of filter paper moistened with 100 mL muddy tap water in 250 mL vials each. All vials were put into growth cabinets. Last weights of the groups were determined at the end of the 7th day. Based on the first and last weights, four-day fresh weight changes were established as $g.g^{-1}$. Twenty germinated seeds per each group were used in weight measurements (Baltepe *et al*., 1982).

Determination of pigment amount: In order to determine pigment amounts, primary leaves of one-week seedlings grown as described in 2.3 above were used. For each group fresh leaf tissue of about 1 g was taken. The leaves were extracted for pigment analysis (Witham *et al*., 1971) and absorbance of each extract was read blindly at 645 and 663 nm wavelengths. To determine absorbance, quartz cubes of 1 cm^3 volume were employed. Chlorophyll (a+b) amounts were calculated from the absorbance values obtained (Witham *et al*., 1971). CE-5502 Scanning Double Beam UV Spectrophotometer was used in absorbance measurements.

Statistical analyses: All the experiments were repeated three times. The findings were statistically analyzed (SPSS 10.0 Windows, Duncan and Kruskal Wallis test), calculating the standard deviation of the mean and conducting variance analyses.

RESULTS AND DISCUSSION

Effects of the aqueous solutions of different concentrations (0, 10^{-5}, 10^{-4}, 10^{-3} and 0.5×10^{-2} M) of ASA on germination activities of cucumber seeds were followed for 3 days. Within this period, measurements were carried out every 12 h. It was found that 0.5×10^{-2} M ASA application significantly prevented germination of the seeds (100% at the end of 12 h; 26.66% at the end of 24 h; 25.60% at the end of 36 h; 15.55% at the end of 48 h; 11.95% at the end of 60 h and 9.27% at the end of 72 h) ($p<0.05$). 10^{-5}, 10^{-4} and 10^{-3} M ASA concentrations did not produce any significant difference in germination ($p>0.05$) (Table 1). Radicle growth of 3-day seedlings was significantly reduced by 0.5×10^{-2} M ASA application (77.82%) ($p<0.05$), while 10^{-5} M ASA application stimulated radicle growth (6.31%). 10^{-4} and 10^{-3} M concentrations of ASA did not influence radicle growth ($p>0.05$) (Table 2).

Results of various growth parameters and chlorophyll (a+b) amounts of the one-week seedlings grown from seeds which were applied ASA for 48 h starting from the swelling time are presented in Table 2 and 3. 0.5×10^{-2} M ASA led to decreases of 80.28, 73.15, 67.56 and 77.12% in the growth of root, hypocotyl, leaf and plant lengths, respectively ($p<0.05$). This concentration of ASA reduced fresh weight increase by 59.68% ($p<0.05$) and chlorophyll content of primary leaves by 22.75% ($p<0.05$). As opposed to 0.5×10^{-2} M ASA, 10^{-5} M ASA application brought about 8.10, 17.44, 9.23 and 8.52% increases in root, hypocotyl and plant lengths and fresh weights of the seedlings, respectively ($p<0.05$). 10^{-3} M ASA application inhibited only the root length increase (12.16%) ($p<0.05$) and chlorophyll (a+b) amount (12.91%) ($p<0.05$) of the plants. 10^{-4} M ASA applications did not produce any effect in terms of the studied features ($p>0.05$). In the present study, a strong phytotoxic effect was observed in high concentrations of ASA (Jung *et al*., 2001). 0.5×10^{-2} M ASA application, in particular, significantly delayed germination. However, applications of lower concentrations did not produce any difference in comparison to the control (Khan and Ungar, 1986; Pancheva *et al*., 1996; Jung *et al*., 2001; K'Opondo *et al*., 2001; Colpas *et al*., 2003; Regiosa *et al*., 2004).

High ASA concentrations were observed to inhibit root growth in 3-day seedlings (0.5×10^{-2} M ASA) and one-week seedlings (0.5×10^{-2} and 10^{-3} M ASA) (Larque-Saavedra, 1978; Pancheva *et al*., 1996). However, 10^{-5} M ASA had a growth increasing effect in both time periods (Yamamoto and Fujii, 1997). Other concentrations were

Table 1: Percentage values of 3 day germination activities of cucumber (*Cucumis sativus* L. Beit Alpha) seeds to which ASA was applied for 48 h

Hours	0 M	10^{-5} M	10^{-4}M	10^{-3}M	0.5×10^{-2} M	p-values
	----------($\bar{x} \pm S\bar{x}$)----------					
12	20±2.13a	25±3.02a	21±2.71a	16±2.16a	0±0.00b	<0.05
24	45±3.51a	51±3.22a	52±3.52a	49±2.12a	33±2.33b	<0.05
36	82±3.54a	88±3.33a	82±2.34a	84±2.07a	61±3.61b	<0.05
48	90±2.28a	94±2.25a	91±2.56a	95±3.24a	76±3.02b	<0.05
60	92±3.57a	96±3.80a	93±2.09a	96±3.82a	81±3.42b	<0.05
72	97±2.44a	96±2.46a	99±2.09a	97±2.70a	88±2.60b	<0.05

a, b: Differences between group means with different letter (s) in the same line are significant ($p<0.05$), n: 100

Table 2: Values pertaining to various growth parameters in seedlings that grew from cucumber (*Cucumis sativus* L. Beit Alpha) seeds to which ASA was applied for 48 h

Growth parameters (cm)	0 M	10^{-5} M	10^{-4}M	10^{-3}M	0.5×10^{-2} M	p-values
	----------($\bar{x} \pm S\bar{x}$)----------					
Radicle length (3 days)	2.255±0.05b	2.3975±0.06a	2.2675±0.05b	2.1525±0.04b	0.5000±0.02c	<0.05
Root length (one week)	10.175±0.28b	10.8200±0.25a	10.2250±0.49b	8.9375±1.01c	2.0056±0.29d	<0.05
Hypocotyl length (one week)	3.725±0.15b	4.1750±0.13a	3.7750±0.25b	3.5500±0.45b	1.0000±0.32c	<0.05
Leaf length (one week)	1.850±0.13a	1.8600±0.15a	1.8500±0.08a	1.8400±0.07a	0.6000±0.12b	<0.05
Seedlings length (one week)	15.760±0.47b	16.8350±0.57a	15.8500±0.37b	14.3375±1.92b	3.6056±0.38c	<0.05

a, b, c, d: Differences between group means with different letter (s) in the same line are significant ($p<0.05$), n: 20

Table 3: Values pertaining to fresh weight and cl (a+b) amounts in one-week seedlings that grew from cucumber (*Cucumis sativus* L. Beit Alpha) seeds to which ASA was applied for 48 h

Parameters	0 M	10^{-5} M	10^{-4}M	10^{-3}M	0.5×10^{-2} M	p-values
	----------($\bar{x} \pm S\bar{x}$)----------					
Fresh weight (g g^{-1})	0.3014±0.01b	0.3271±0.01a	0.2987±0.02b	0.2975±0.02b	0.1215±0.04c	<0.05
Chlorophyll (a+b) amounts (mg g^{-1}. wet weight)	1.200±0.05a	1.194±0.06a	1.193±0.06a	1.045±0.05b	0.927±0.05c	<0.05

a, b, c: Differences between group means with different letter (s) in the same line are significant ($p<0.05$)

observed to have no effect on root growth (Jung *et al*., 2001; Regiosa *et al*., 2004). Furthermore, in the group that was applied 0.5×10^{-2} M ASA, roots remained short in a majority of the seedlings, while primary root never developed and instead, adventive root formation was more prominent in a small portion (Colpas *et al*., 2003). The high concentration (0.5×10^{-2} M) was observed to have an inhibitive effect on hypocotyl growth in one-week seedlings, whereas the low concentration (10^{-5} M) had an augmentative effect thereof. Likewise, 0.5×10^{-2} M ASA had a marked inhibitive effect on leaf length increase, while other applications were seen to be ineffective (Pancheva *et al*., 1996). Leaf edges of the seedlings to which 0.5×10^{-2}M ASA was applied were observed to have marked chlorosis. It was seen that, in general, 0.5×10^{-2} M ASA application had a significantly inhibitive effect (Shettel and Blake, 1983) and 10^{-5}M ASA application had an augmentative effect on plant growth, while other applications were not different from the control. 0.5×10^{-2} M ASA application exercised an inhibitive effect (Jung *et al*., 2001) and 10^{-5} M ASA application had an augmentative effect (Jung *et al*., 2001) on fresh weight increase in one-week seedlings. The antitranspirant effect of ASA can be an important factor in fresh weight changes (Larque-Saavedra, 1978). 0.5×10^{-2} and 10^{-3} M ASA applications were found to bring about a decline in chlorophyll (a+b) amount in the leaves taken from one-week seedlings (Pancheva *et al*., 1996).

The effect of SA on the regulation of plant growth has been considered significant for a long time (Raskin, 1992, 1995; Çanakci, 2003; Pancheva *et al*., 1996). Present results show that dose-response correlation of ASA, which is known to hydrolyze to SA in aqueous solutions (Mitchell and Broadhead, 1967), is clear: lower doses encourage growth, while higher doses inhibit it. These effects are marked on hypocotyl and root growth. The inhibitive effect observed in high concentrations can be attributed to toxic stress, but we do not know by which mechanism the stimulating effect can be explained. This may be associated with the (oxine-like) regulatory effect of SA on cell growth and division (Kling and Meyer, 1983). Similarly, the decreases in chlorophyll amounts in high ASA concentrations may be attributed to the toxic effect. This decrease caused by acetylsalicylic acid in chlorophyll amount was claimed to be a result of the prevention of chlorophyll biosynthesis or acceleration of chlorophyll destruction or both (Yang *et al*., 2002). These issues can be clarified only through further studies.

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