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EFFECT OF SALICYLIC ACID ON THE EXPRESSION OF HEAT-SHOCK PROTEINS AND MORPHOLOGY OF POTATO (*SOLANUM TUBEROSUM* L.) PLANTS UNDER HEAT STRESS *IN VITRO*

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UTICAJ SALICILNE KISELINE NA EKSPRESIJU PROTEINA TOPLOTNOG STRESA I MORFOLOŠKE OSOBINE BILJAKA KROMPIRA (*SOLANUM TUBEROSUM* L.) U USLOVIMA TOPLOTNOG STRESA *IN VITRO*

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ABSTRACT

The potato, *Solanum tuberosum* L., is the fourth most important crop in the world. Most of the commercial potato cultivars were developed for growing in temperate climate regions and they are highly sensitive to elevated temperatures. As a result of global warming over next 60 years, significant potato yield losses in the range of 18 to 32% are predicted. These losses can be reduced with adaptations in production methods, such as terms of planting time and use of heat tolerant cultivars. Besides, exploration of compounds that may enhance potato heat tolerance, such as salicylic acid (SA), is an alternative approach to cope with adverse effects of high temperatures on potato growth and yield. The major aim of this study was to investigate effects of SA on the basal heat tolerance in *Solanum tuberosum* L. Potato heat tolerance correlates with level of small heat-shock proteins (sHSP) accumulation, therefore SA effect on this group of HSPs, as well as HSP101 and Cu/ZnSOD, was investigated. Besides, it was interesting to investigate SA effects on morphological parameters related to plant growth and development. Experiments were conducted *in vitro* since this experimental setup allowed investigation under strictly controlled conditions, with variation of only selected factors – temperature and SA concentration. Experimental procedure based on potato single-node stem cuttings (SNC) exposure to standard growth temperature (23 °C), excessively elevated temperature (45 °C, 6 h; short-term HS) or prolonged exposure to moderately elevated temperature (35 °C, 20 days; long-term HS) has been established. Similar percentage of plants developed from SNC at 23 °C and during recovery period after exposure to short-term HS, while long-term HS substantially reduced the number of plantlets developed from SNC implying that in potato prolonged, moderately elevated temperatures can cause more devastating effects then excessively elevated ones during short exposure. SA in a range of concentrations from 10^{-6} M to 10^{-4} M stimulated development of plants from SNC under long-term HS in all investigated potato cultivars, with the exception of cultivar ‘Marabel’ which did not respond on SA treatment. These findings indicate that SA may enhance basal thermotolerance in potato cultivars/genotypes, as well as variation in genotypes´ sensitivity to SA. SA pretreatments, in the range of concentrations from 10^{-6} M to 10^{-4} M, did not affect internode initiation and elongation either under standard or elevated temperatures, while SA treatments in the same range of the concentrations stimulated

these processes under both short-term and long-term HS. Both SA pretreatments and SA treatments, in the range of concentrations from 10^{-6} M to 10^{-4} M, strongly inhibited root initiation and elongation at standard growth temperature and after short-term HS, while SA treatments did not show prominent inhibitory effect on root elongation in the same range of the concentrations under long-term HS. The results suggest interaction between SA and HS in the process of root elongation, possibly depending on duration and intensity of HS, as well as on duration of exposure to SA. SA pretreatment had stimulatory effect on HSP17.6 accumulation, while all applied SA concentrations inhibited accumulation of chloroplast HSP21 in investigated potato genotypes when exposed to 35 °C or 45 °C for 6 h. Relatively low SA concentration (10^{-6} M) promoted accumulation of HSP101 in potato, moderate (10^{-5} M) did not affected abundance, while high concentration (10^{-4} M) reduced accumulation of this protein under 45 °C-treatment. These findings indicate significant differences in SA-mediated regulation of HSP17.6, HSP21 and HSP101 expression under HS. Pretreatment with only 10^{-5} M SA had strong stimulatory effect on Cu/ZnSOD accumulation at 23 °C in leaves of investigated potato cultivars. Stimulatory effect of 10^{-5} M SA was accentuated at 35 °C, while slight increase in abundance of this protein has been observed at 45 °C in most potato genotypes. These findings indicate that stimulatory effect of SA on Cu/ZnSOD accumulation in potato depends on both temperature treatment and SA concentration.

Key words: *Solanum tuberosum* L., salicylic acid, heat stress, heat shock proteins, HSP, Cu/ZnSOD

Scientific field: Biology

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REZIME

Krompir, *Solanum tuberosum* L., je četvrta po značaju kulturna biljka u svetu. Najveći broj sorti krompira je razvijen za gajenje u umerenim klimatskim zonama i izuzetno su osetljive na visoke temperature. Kao rezultat globalnog zagrevanja u narednih 60 godina, predvidja se značajan pad u prinosu krompira izmedju 18% i 32% koji bi se mogao smanjiti prilagođavanjem poljoprivredne prakse u smislu promene u vremenu sadjenja i lokacija, kao i korišćenju sorti otpornih na visoke temperature. Primena jedinjenja koja pospešuju otpornost krompira na toplotni stres predstavlja drugi način da se prevaziđu negativni efekti visokih temperatura na rast i prinos ove kulturne biljke. Osnovni cilj ovog rada bio je ispitivanje uticaja salicilne kiseline (SA) na otpornost *S. tuberosum* prema toplotnom stresu. Otpornost krompira na toplotni stres je u korelaciji sa nivoom akumulacije malih HSP (sHSP), pa je stoga ispitivan efekat SA na ekspresiju ove grupe proteina, kao i HSP101 i Cu/ZnSOD. Pored toga ispitivan je uticaj SA na morfološke osobine krompira u uslovima toplotnog stresa. Korišćenje kulture *in vitro* omogućilo je izvodjenje eksperimenata pod strogo kontrolisanim uslovima, uz variranje samo dva faktora – temperature i koncentracije SA. Eksperimentalna procedura se zasnivala na izlaganju jednonodalnih odsečaka stabla krompira (SNC) različitim temperaturnim tretmanima, uključujući standardnu temperaturu za uzgoj *in vitro* (23 °C, 20 dana) kratkotrajni tretman ekstremno visokom temperaturom (45 °C, 6 h – kratkotrajni toplotni stres) i dugotrajni tretman umereno visokom temperaturom (35 °C, 20 dana; dugotrajni toplotni stres). Sličan procenat biljaka razvio se iz SNC na 23 °C i tokom perioda oporavka po kratkotrajnom toplotnom stresu, dok je dugotrajni tretman umereno visokom temperaturom značajno redukovao razvoj biljaka iz SNC ukazujući da dugotrajno delovanje umereno visoke temperature ima veći uticaj na razvoj biljaka nego kratkotrajni tretman ekstremno visokom temperaturom. SA (10^{-6}-10^{-4} M) je stimulisala razvoj biljaka iz SNC tokom dugotrajnog toplotnog stresa kod svih ispitivanih sorti krompira, sa izuzetkom sorte Marabel koja nije reagovala na SA. Dati rezultati ukazuju da SA može pospešiti otpornost krompira na toplotni stres, ali da postoji značajna razlika u osetljivosti sorti/genotipova na delovanje SA. SA pretretmani (10^{-6}-10^{-4} M) nisu imali efekta na inicijaciju i izduživanje internodija u uslovima standardne temperature gajenja, kao i tremana visokim temperaturama, dok su SA tretmani stimulatorno delovali na date procese u uslovima kako kratkotrajnog, tako i

dugotrajnog toplotnog stresa. SA pretretmani i SA tretmani (10^{-6}-10^{-4} M) su značajno inhibirali inicijaciju i izduživanje korenova u uslovima standarne T gajenja, kao i kratkotrajnog toplotnog stresa, dok SA tretmani nisu pokazali značajniji inhibitorni efekat na elongaciju korenova u uslovima dugotrajnog toplotnog stresa. Ovi rezultati ukazuju na interakciju SA i toplotnog stresa u procesu izduživanja korenova koja zavisi of dužine toplotnog tretmana, kao i dužine izlaganja biljaka SA. SA pretretman je stimulatorno delovao na akumulaciju HSP17.6, dok je uočeno smanjenje zastupljenosti HSP21 u listovima biljaka krompira pri izlaganju temperaturama od 35 °C ili 45 °C 6 h. Relativno niska koncentracija SA (10^{-6} M) je stimulisala akumulaciju HSP101 krompira, srednja (10^{-5} M) nije imala efekta, dok je visoka koncentracija (10^{-4} M) smanjila zastupljenost HSP101. Navedeni rezultati ukazuju na značajne razlike u SA-posredovanoj regulaciji ekspresije HSP17.6, HSP21 and HSP101 u uslovima toplotnog stresa. Samo pretretman sa 10^{-5} M SA je imao jak stimulatorni efekat na akumulaciju Cu/ZnSOD u listovima svih ispitivanih sorti krompira na 23 °C. Stimulatorni efekat 10^{-5} M SA se pojačao u uslovima 35 °C-toplotnog stresa, dok je mali porast u zastupljenosti proteina uočen na 45 °C. Dati rezultati ukazuju da stimulatorni efekat SA na akumulaciju Cu/ZnSOD krompira zavisi kako od temperaturnog tretmana, tako i primenjene koncentracije SA.

Ključne reči: *Solanum tuberosum* L., salicilna kiselina, toplotni stres, heat-shock proteini, HSP, Cu/ZnSOD

Naučna oblast: Biologija

Uža naučna oblast: Fiziologija biljaka

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ABBREVIATIONS

ABA	abscisic acid	**MeSA**	methyl salicylate
ASA	acetylsalicylic acid	**mRNA**	messenger ribonucleic acid
BA	6-benzylaminopurine	**NADPH**	nicotinamide adenine dinucleotide phosphate
BM	basal medium	**NFDM**	non-fat dry milk
CMD	cellular membrane damage	**OEC**	oxygen evolving complex
DNA	deoxyribonucleic acid	**PAL**	phenylalanine ammonia lyase
EDTA	ethylenediaminetetraacetic acid	**PBS**	phosphate buffered saline
ELA	electrolyte leakage assay	**PGR**	plant growth regulator
ET	ethylene	**PSII**	photosystem II
FAO	Food and Agricultural Organization	**PVDF**	polyvinylidene di fluoride
HS	heat stress	**ROS**	reactive oxygen species
HSE	heat shock element	**RuBisCO**	ribulose-1,5-bisphosphate carboxylase/oxygenase
HSF	heat shock transcription factor	**SA**	salicylic acid
HSP	heat shock proteins	**SAG**	salicylic acid O-β-glucoside
IAA	indole-3-acetic acid	**SAGT**	salicylic acid glucosyltransferase
ICS	isochorismate synthase	**SAR**	systemic acquired resistance
JA	jasmonic acid	**SDS-PAGE**	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
kDa	kilodaltons	**SGE**	salicyloyl glucose ester
Kin	kinetin	**sHSP**	small heat shock protein
LAI	leaf area index	**SNC**	single-node stem cuttings

CONTENTS

1. INTRODUCTION

1. INTRODUCTION

The potato, *Solanum tuberosum* L., is the fourth most important crop in the world in terms of acreage grown, yield and value of the crop (FAO, 2009). More important are only wheat, corn and rice. Potato is not only a cash crop but also a substitute of food crop next to rice and wheat. It is also an important crop in terms of dry matter production (2.2 t ha^{-1}), energy (216 MJ ha^{-1} day^{-1}) and nutrition (Beukema and van der Zaag, 1990). The suitability of the potato for a wide range of growing conditions coupled with its ability to produce large quantities of nutritious food has driven a rapid increase in production on an estimated 192.000 km^2 of farmland globally, with production of more than 364 million tonnes per annum (FAOSTAT, 2012).

The potato crop is threatened by abiotic stress conditions such as cold, frost, heat and drought (Bradshaw and Ramsay, 2009). The adverse effects of high temperatures on potato growth and yield are numerous and include: the acceleration of stem growth with assimilate partitioned more toward the stem, the reduction of photosynthesis and increase of respiration, reduction of root growth, inhibition of tuber initiation and growth, frequent tuber disorders, reduction of tuber dry matter and increase of glycoalkaloid level (Struik, 2007b). The devastating effects of the heat stress on plant growth may directly outcome from increasing temperatures in tissues and organs, or indirectly from water deficits due to high transpiration rates that increases exponentially with temperature elevation (Hall, 2001). As a result of global warming over next 60 years, significant potato yield losses in the range of 18 to 32% are predicted (Hijmans, 2003). However, these losses can be reduced to 9-18% with adaptations in production methods, such as terms of planting time and use of heat tolerant (HT) cultivars. Some potato cultivars have already been developed using conventional breeding to enhance heat tolerance for warmer climate cultivation (Susnoschi *et al.*, 1987; Veilleux *et al.*, 1997; Minhas *et al.*, 2001; Levy *et al.*, 2001; Levy and Veilleux, 2007). Besides, exploration of compounds that may enhance potato heat tolerance, such as salicylic acid, is an alternative approach to cope with adverse effects of high temperatures on potato growth and yield.

1.1. The Effects of Global Warming on Crop Production

Climatic conditions are changing significantly at regional and global scales and the climate at the end of this century will be substantially warmer than that of the past century (Naylor *et al*., 2007). There is broad consensus among some 20 global climate models considered by the Intergovernmental Panel on Climate Change (IPCC) that ambient temperature around the globe may increase between 1.1 and 6.4 ^{o}C with the doubling of atmospheric carbon dioxide by the end of the century (Meehl *et al*, 2007). According to IPCC, there was a 0.5 ^{o}C increase during the past 100 years and it is expected that our earth will be 0.2 ^{o}C warmer per decade for the next two decades.

In plants, optimum temperature is essential for reproduction and maximum yield (Cheikh and Jones, 1994; Keeling *et al*., 1994). High temperature affects morphological, biochemical and physiological processes in plants. The major effects entail scorching of aerial plant parts, sunburn of branches and stems, leaf abscission and senescence, acceleration of developmental stages, inhibition of shoot and root growth, abortion of seed development, reduced grain filling and yield (Ismail and Hall, 1999; Stone, 2001; Wahid *et al*., 2007a). The extent to which heat stress causes damage on plants is a complex issue. It depends on the intensity, duration and rate of temperature increase, as well as other environmental conditions, such as when the high temperature occurs (during the day or the night) and where it occurs (in the air or the soil) (Porter, 2005; Sung *et al*., 2003).

Global warming will have a great impact on agriculture. A gradual increase in temperature is likely to change cropping pattern, growth and economic yield of many important crops (Wollenweber *et al*., 2003). Over half a billion people are at extreme risk to the impacts of climate change, and six in ten people are vulnerable in a physical and socioeconomic sense (Global Humanitarian Forum, 2009). Together with local overpopulation and poor land and water management, climate change is already responsible for causing hunger and malnutrition for some 45 million people worldwide as a result of reduced yields of cereals, fruits, vegetables, livestock and dairy, and cash crops like cotton and fish (Global Humanitarian Forum, 2009). Abiotic stresses cause a reduction of average yields of most major crops by more than 50% and annual losses estimated at billions of dollars (Mittler, 2006).

1.2. Potato - *Solanum tuberosum* L.

Potato is an important tuber crop, mainly used as vegetables, which belongs to the family Solanaceae, genus *Solanum*, section *Petota*, subsection *Potatoe* (Hawkes, 1990). The genus *Solanum* is very large, containing 1500-2000 species. Two other well-known species in this genus are the eggplant (*S. melongena*), tomato (*S. lycopersicum*) and black nightshade (*S. nigrum*). Other cultivated plant species of the Solanaceae family include pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabacum*).

The subsection *Potatoe* includes seven cultivated potato species, as well as about 200 wild tuber bearing potato species. Several taxonomical studies suggest that wild potato species are well spread from North to South America and highly diverse (Hawkes, 1990; Spooner and Hijmans, 2001). The ploidy level ranges from diploid (2n=2x=24) to hexaploid (2n=6x=72) in which the majority of diploid species is self-incompatible and tetraploids and hexaploids are self-compatible allopolyploids that exhibit disomic inheritance (Hawkes, 1990). The other subsection of the section *Petota*, *Estolonifera*, includes the non-tuberizing species.

According to Hawkes (1978b), the commercial potato, as we know it today, belongs to a single species, *S. tuberosum* L. *S. tuberosum* is the tetraploid species (2n = 4x = 48 chromosomes), and may be divided into subspecies *tuberosum* and *andigena*. *S. tubersosum* ssp. *andigena* is the most widely grown in South America. Tubers of this subspecies have deep eyes, are often pigmented and are produced during short day lengths. Most of the commercially important cultivars of potato, *S. tuberosum* L. ssp. *tuberosum*, have been bred in the temperate climates of Europe and North America, where the centers for potato breeding and research flourished. This potato subspecies has been adapted to long days, cool climates and is generally adversely affected by high temperature (Hawkes, 1978a).

1.2.1. Morphology

The potato is an annual herbaceous, dicotyledonous plant. Due to its asexual reproduction through tubers, it may also act as a perennial plant. Besides, it can be grown from the seeds. Germination is epigeal, i.e. the cotyledons are borne above ground by elongation of the hypocotyl (Cutter, 1978).

The aboveground stem is herbaceous and erect in the early stages (Fig. 1). Several auxiliary branches are usually produced. The stems are round to sub-triangular or quadrangular in the cross section. Leaves are alternate in a counterclockwise spiral around the stem. Leaves are compound and leaflets vary with the variety. Very small secondary leaflets grow between the primary leaflets of the compound leaf. Stomata appear on both surfaces of the leaflets, but are more numerous on the lower surface.

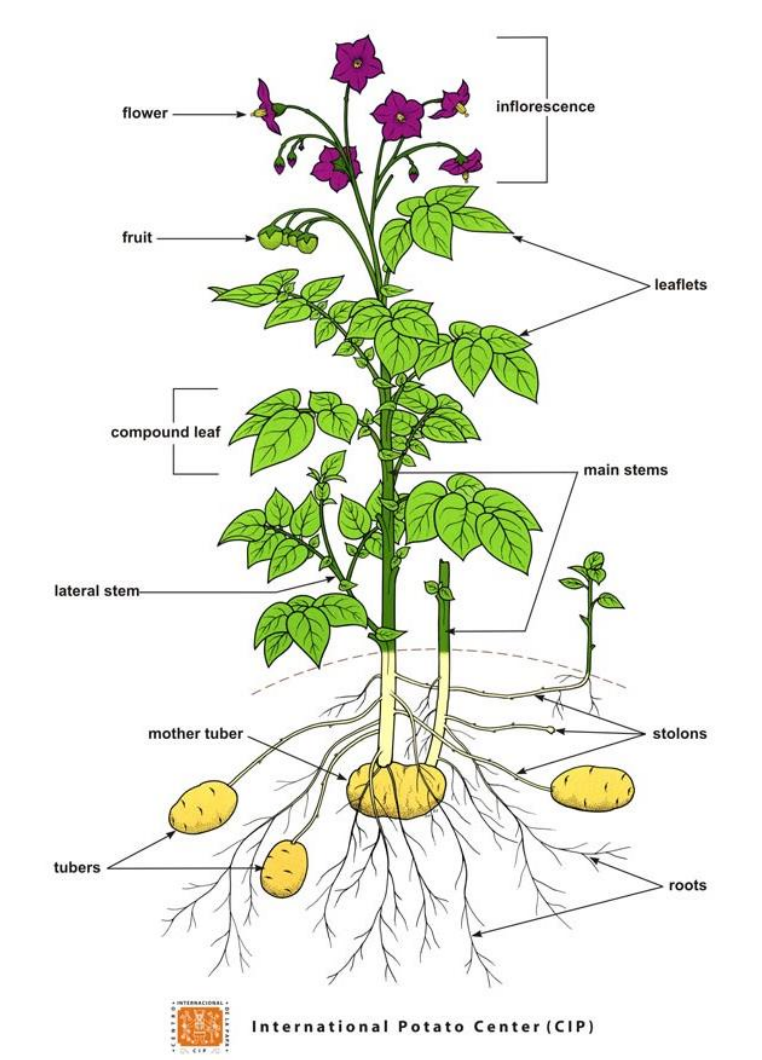

Fig. 1 Morphology of potato plants. According to International Potato Center (http://cipotato.org/potato/how-potato-grows/).

Stolons are modified, plagiotropic shoots or stems with elongated internodes and rudimentary leaves (scales) that develop from the basal, below ground stem nodes of

potato (Struik, 2007a). Tubers, which can be described as swollen stolon parts bearing axillary buds (eyes), are formed at the tip of the stolons. The development of stolons depends largely on certain environmental conditions, especially the exclusion of light (Smith, 1977). According to Steyn (1999) tuber initiation starts when the leaf area index (LAI) is approximately 1.5 to 2 (LAI = Leaf area (m^2) / Soil area (m^2). LAI can be influenced by factors such as plant density, irrigation, fertilization etc. As the LAI increases tubers increase in size due to the translocation of carbohydrates formed through the process of photosynthesis. Photoperiod and temperature are the most important factors influencing tuberization in potato. Concerning tuberization, cultivars of *S. tuberosum* ssp. *tuberosum* are adapted to long-day conditions (12 to 18 hours), while cultivars of ssp. *andigena* demand short days (12 to 13 hours) (Ewing and Struik, 1992). The critical day-length for tuber induction, as well as photoperiodic response, varies in different potato genotypes (Snyder and Ewing, 1989). Air temperatures strongly influence tuber induction in potato; day temperatures below 30 °C and night temperatures below 20 °C promote tuberization (Ewing, 1995). Air temperatures higher than these inhibit tuberization both under long-day and short-day conditions (Jackson, 1999). According to Slater (1963), tuber initiation depends on both day length and temperature. During the short days moderate temperatures (20 °C day / 14 °C night) enhance tuber initiation.

1.2.2. Growth and Development

Growing from seeds is rarely used in practice, except for the selection of new potato varieties. *S. tuberosum* ssp. *tuberosum* is tetraploid, which may possess 4 different alleles per locus and, therefore, the high level of heterozygosity in F1 generation. This can cause significant change of desirable cultivar characteristics in the progeny. The growth and development of the potato plant, vegetatively propagated through tubers, can be divided into five distinct growth stages (Rowe, 1993):

- Growth stage I - Sprout development. Sprouts develop from eyes on seed tubers and grow upward to emerge from the soil. Roots develop at the base of the sprouts. During this stage the seed tuber is the only source of energy for growth, as photosynthesis has not yet begun.

- Growth stage II - Vegetative growth. After emergence of the sprouts, leaves and stems develop from aboveground nodes. During this stage photosynthesis begins to produce carbohydrates for growth and development. At this stage the plant uses still energy obtained from the seed tuber. Roots and stolons develop from underground stem nodes. This stage lasts until the tubers start to develop at the tips the underground stolons.
- Growth stage III - Tuber initiation. Tubers form at the end of stolons and are very small at this stage. This stage is a relative short period and depends on climatic conditions, nutrition and variety (Steyn, 1999). In South Africa, this stage is usually longer than in European countries, where the temperatures are moderate.
- Growth stage IV - Tuber bulking. During this stage tuber enlarge and gain weight. Cells in the tuber expand up to 18 times their normal size as a result of the accumulation of water, starch and nutrients. Tuber bulking occurs in a nearly linear fashion if growth is optimal. Tubers become the dominant site for the deposition of carbohydrates and mobile inorganic nutrients within the plant.
- Growth stage V - Maturation. Shoots turn yellow and lose leaves, photosynthesis gradually decreases, the tuber growth rate slows, and shoots eventually die. Dry matter of tubers reaches a maximum and skins of tubers set and thickens.

According to Struik (2007b), development of tubers can be separated into several steps, including tuber induction, tuber initiation, tuber set, tuber bulking and tuber maturation. During tuber induction, plant integrates environmental and internal signals and translates them into determination for tuber formation, although there are still no visible signs of tuber formation. Tuber initiation is characterized by first swelling of stolon tips into tuber initials. During tuber set most of the initials, which will grow into sizable tubers, are formed. Tuber bulking is characterized by enlargement of tubers. During tuber maturation, tuber skin forms and suberize. At the end of this stage, tuber dormancy begins.

1.2.3. Potato Nutritional Value and Food Security

Potato is an ideal agricultural staple for human consumption, considering its high starch and vitamin content (Fernie and Willmitzer, 2001). Potatoes are particularly useful as a source of energy. Newly harvested potatoes contain approximately 80% water and 20% dry matter, with 60-80% of dry matter as carbohydrate, predominantly starch (Camire *et al*., 2009). Tubers also contain high quality proteins, vitamins and dietary fibers, but only little fat. Some plant secondary metabolites in the tubers (calystegine alkaloids, antioxidative phenolic compounds, lectins) may be also beneficial for the human diet (Friedman, 2006). On the other hand, potato glycoalkaloids are secondary metabolites that at certain levels may be toxic to humans and new cultivars are screened for their content before release for the commercial use (Friedman, 2006). Potatoes are a good source of ascorbic acid (vitamin C) and B vitamins, especially niacin, thiamine, vitamin B6 and riboflavin (Horton and Sawyer, 1985; Bajaj, 1986). Vitamin C is the predominant vitamin in potatoes, with one medium potato (boiled with skin) containing 18.4 mg, which is 20% and 25% of the Recommended Daily Allowance (RDA) for adult men and women, respectively (Institute of Medicine US, 2000). The appreciable quantity of lysine in potato makes it a valuable supplement to cereals, which are limited in lysine. Potato is also a good source of phosphorous and magnesium, a moderate source of iron and rich in potassium (Horton and Sawyer, 1985). In addition to vitamins and minerals, potatoes also contain phytochemicals with antioxidant potential, which can vary in amount and composition among varieties (Brown, 2005).

"Since the potato was first established in Europe from South America in the 16th Century, it was at first mistreated, then loved and finally feared because of its poor disease resistance" stated Lehtonen (2009). Between 1845 and 1848, The Irish potato famine, killed one million people and led 2.5 million people to emigrate, making it one of the worst famines in modern European history (FAO, 2009). Nowadays, the importance of the potato as a food of the world is increasing. The failure of cereal surpluses to meet international demands together with rapidly increasing food prices has increased the requirements for potato as an easily grown and nutritious food source. Adding new land for potato production, reducing losses due to both biotic and abiotic stresses, and increasing crop productivity, are some of the measures that will increase

food security. The annual global production approaches 370 million tons, with China accounting for more than 20% of the world production and 25% of total acreage (FAOSTAT, 2012). The year 2008 was designated The International Year of the Potato by the Food and Agricultural Organization of the United Nations (FAO, 2009).

1.2.4. Climatic Requirements for Growing Potatoes

Knowledge of the climatic requirements of the potato and its physiological responses to the environment is extremely important to help growers produce high yields with good tuber quality under site-specific atmospheric conditions. Potato is a cool-season crop with an optimal temperature for plant and tuber growth within the range 15-20 °C (Ewing, 1981; Manrique, 1995). It grows optimally under relatively cool conditions and the formation and tuber bulking depends mainly on day and night temperatures, to enable metabolites produced during daytime to accumulate in the tuber during night (Hooker, 1981). Heat stress drastically affects growth and development of potato and has a detrimental impact on its yield modifications of the sink-source relationships (Mendoza and Estrada, 1979; Ewing, 1981). An agrometeorological model is presented based on carbon dioxide assimilation maximum rates for C3 plants, fraction of photosynthetically active radiation (PAR), air temperature and photoperiod duration (Pereira *et al*., 2008). It is well known that production and the quality of tubers in potato, as a C3 plant, is affected by factors such as air temperature, and the length of the light rays which determines rate of photosynthetic CO_2 assimilation. Also, differences in weather patterns, soil, seed potato quality, suitability of cultivars for the climatic conditions, agricultural management can cause variability in the productivity and quality of potato tuber crop ranging from 4 t ha^{-1} to 56 t ha^{-1} (Bowen, 2003).

1.2.5. The Effects of Temperature on Potato Crop

Sarquis *et al*. (1996) stated that the magnitude of the effect of elevated temperatures on potato growth and final yield is determined by an intricate interaction between soil temperature, air temperature, solar radiation flux density, and photoperiod duration. The potato buds are especially sensitive to alterations in temperature. Therefore both low and high temperatures may limit growth and sometimes a severe damage may lead to

death of potato plants. The highlands like the Andes and hillsides of East Africa and Central Asia, where potato is grown, are much more affected by the drastic changes in the night temperature. In these regions, frost and freezing can cause damage to potato not only at the plant early growth stages, but during the entire growing period (Watanabe, 2002). High temperatures, which prevail during year in many developing countries, also represent important limiting factor in potato production (Dodds *et al.*, 1990). Increased temperatures are detrimental to tuber formation and during the late growth stage may cause problems such as necrosis (brown spots) inside the tubers (Zotarelli *et al.*, 2012). Besides, high temperatures can cause secondary growth of tubers and chain-tuber formation, growth cracks and heat sprouts (Struik, 2007b).

Reductions in leaf area, tuber number and tuber weight have been reported as symptoms of elevated temperatures during the growing season of potato plants (Menzel, 1985). High temperatures are also causing excessive haulm growth at the expense of the tubers (Ewing, 1981), increased rate of respiration (Sale, 1974), reduced dry matter accumulation and delayed tuber initiation and maturity (Amadi, 2005). Temperature rise to 30°C increases the respiration process and lessen production (Sarquis *et al*., 1996). The limited production of potato in warm climates is affected by several factors. Firstly, elevated temperatures cause increase in photorespiration and inhibition of photosynthesis, resulting in a shortage of photo assimilates in tubers (Berry and Bjorkman, 1980). Secondly, high temperatures alter photosynthate partitioning in favor of above-ground plant parts over tubers (Gawronska *et al*., 1992). At high temperatures, new leaves may effectively compete for limited assimilates because of their proximity to source leaves. It is possible that photosynthates in leaves are partly utilized during recovery from heat stress. Thirdly, high soil temperatures may directly decrease the starch content of potato tubers by inhibiting the conversion of sugars into starch (Krauss and Marschner, 1984). Geigenberger *et al*. (1998) demonstrated that an elevated temperature (37°C) inhibited the activity of adenosine diphosphate (ADP)-glucose pyrophosphorylase, an enzyme that is involved in starch synthesis in the tubers. Extremely high temperatures (42°C) are lethal and cause irreversible damages including protein denaturation and membrane leakage (Blum, 1988) and a significant loss of tuber yield and quality (Levy, 1984).

1.2.6. Breeding and Biotechnology of Heat Tolerant Potato

The adverse effect of high temperature on tuber yield and quality of potato is a major obstacle for potato production in hot regions, and local breeding of heat tolerant cultivar has been necessary to improve potato crops in a hot environment (Levy, 1984). Genetic diversity of the *S. tuberosum* species represent an opportunity for researchers to obtain varieties of potatoes with ability to tolerate heat stress and give better productivity and quality of this tuber crop. Although *S. tuberosum* ssp. *tuberosum* is extensively adapted to the conditions prevailing in temperate climates, namely, moderate day temperatures, cool nights, and long days, certain genotypes have the capacity to initiate tubers at high temperatures (Ewing *et al*., 1987). Variations in the DNA sequence of genes and their regulatory regions underlie most of the phenotypic variation that has been exploited in modern crops (Bryan *et al*., 2000; Masouleh *et al*., 2009).

Conventional breeding methods, although time consuming and laborious, have the potential to develop crop varieties with environmental stress tolerance (Cullis and Murray, 1991). Some potato cultivars, such as Arma, Ori, Idit, Zohar and Zahov, have already been developed using conventional breeding methods to enhance heat tolerance for warmer climate cultivation (Susnoschi *et al*., 1987; Levy *et al.*, 2001; Minhas *et al*., 2001). Wild *Solanum* spp., frequently characterized by good disease resistance and/or environmental stress tolerances, represent valuable genetic resources (Spooner and Hijmans, 2001). However, most cultivated potatoes are tetraploid, while wild *Solanum* spp. ploidy levels include diploids (2n = 2x = 24) triploids, tetraploids, pentaploids, and hexaploids (2 n = 6x = 72) (Hijmans *et. al*, 2007). Tetraploid cultivated potato cannot easily be crossed with many wild potatoes and the high degree of sterility hinders the progress of conventional breeding. This limits interspecies crosses and favors potato breeding within the cultivated species. Nevertheless, some complex potato hybrids were derived from crosses between *S. tuberosum* cv. Atlantic and 2*n* pollen-producing selections of 11 diploid hybrids (Veilleux *et al*., 1997). The diploid hybrids, obtained from crosses between dihaploid *S. andigena* and heat tolerant *S. berthaultii*, *S. chacoense* or *S. microdontum*, contributed good tuberization potential (tuber number, tuber weight and percent plants producing tubers) under heat stress to the progeny (complex potato hybrids).

Following the development of biotechnology, genetically modified varieties of potatoes have also been produced (Bradshaw and Ramsay, 2009), as well as somatic hybrids by protoplast fusion to obtain interspecies hybrids (Laurila *et al*., 2001). In order to reduce the proportion of the wild species genome from further generations, interspecies hybrids were fused with *S. tuberosum* to obtain second generation somatic hybrids (Rokka *et al*., 2005). Because of the recent expansion of potato cultivation to subtropical, semiarid and arid regions, the need has arisen to heat-tolerant cultivars (Swaminathan and Sawyer, 1983). The majority of research to date using the *Arabidopsis* as a model has advanced our understanding of plant temperature perception and response to heat stress; however, further application of this knowledge towards crops to improve their thermotolerance still remains to be explored (Suzuki *et al*., 2011).

1.3. Salicylic Acid (SA)

Salicylic acid is an endogenous plant growth regulator and signaling molecule involved in defense responses of plants to the presence of pathogens and abiotic stress. SA and related compounds belong to a diverse group of plant phenolics. It is a large group of secondary metabolites produced by plants when exposed to the invasion of some microorganisms or insects, one of the means to defend plants or control over the territory against the competition.

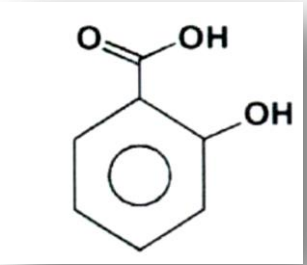

Fig. 2 Structure of salicylic acid (synonym: *ortho*-hydroxy benzoic acid): $C_6H_4(OH)CO_2H$.

Salicylates from plant sources have been used in medicine since ancient times. For instance, ancient Egyptians and Greeks were using willow bark to ease fever and headache. The small amount of glucoside of salicyl alcohol, salicilin, was isolated from willow bark for the first time in 1828 in Münich. Ten years later active ingredient of willow bark was identified and named salicylic acid, from the Latin word *Salix* for a willow tree, by Raffaele Piria. The first commercial production of synthetic SA began in Germany in 1874. Aspirin, a trade name for a close SA analog acetylsalicylic acid (ASA), was introduced by the Bayer Company in 1898 and rapidly became one of the most popular remedy in the world during the 19th century. Despite the fact that aspirin was not identified as a natural product, it is widely used by many plant scientists in their experiments. Acetylsalicylic acid (ASA) undergoes spontaneous hydrolysis to SA and exogenously applied it is rapidly converted to SA (Popova *et al.*, 1997).

1.3.1. Plant Salicylates

Salicylic acid is broadly distributed in kingdom Plantae. A survey of SA in leaves and reproductive structures of angiosperms confirmed the ubiquitous distribution of this compound. However, basal SA level considerably vary among species. In barley, soybean, rice, crabgrass, the levels of SA has been found to be approximately 1 μg g^{-1} fresh weight (Tounekti *et al.*, 2013), in *A. thaliana* basal level of this compound vary between 0.25 μg - 1 μg g^{-1} FW (Brodersen *et al.*, 2005), while potato might comprise up to 10 μg of SA g^{-1} FW (Navarre and Mayo, 2004). The highest levels of SA have been found in the inflorescence of several spice herbs and thermogenic plants (Raskin *et al.*, 1990).

Many different types of SA conjugates have been found in plant species. They were mainly formed by methylation, glucosylation and less frequently by esterification. Large amounts of SA glucosides were detected in *Helianthus annuus*, oat and bean roots (Yalpani *et al.*, 1992 a, b). Methyl salicylate was found in the leaves of oats, red clover, tobacco, and in the volatile fractions of fruits (plum, strawberry, black cherry and tomato) (Lee *et al.*, 1995). SA also forms conjugates with amino acids. Salicyloil aspartic acid was identified in wild grapes, but its physiological function is not known (Silverman *et al.*, 1995).

Potato is plant with high basal level of SA, about 100-fold higher than the levels detected in another *Solanaceae* species - tobacco (Coquoz *et al.*, 1995). Although most of the SA is present in the bound, physiologically inactive form, SA 2-O-β-D-glucoside, levels of the active, free SA are higher than those found in the leaves of many other plant species (Navarre and Mayo, 2004). Relatively high levels of free SA are probably responsible for some differences in potato SA-mediated stress response compared to *Arabidopsis* or tobacco, such as significant basal levels of SA-inducible *PR-1* (pathogenesis related gene 1) present in potato plants in the absence of stress (Navarre and Mayo, 2004).

1.3.2. Salicylic Acid Biosynthetic Pathways

SA is synthesized in plants through two pathways that utilize different precursors and take places in different cellular compartments (Tounekti *et al.*, 2013). First is phenylalanine ammonia-lyase (PAL) pathway starting in the cytoplasm from phenylalanine (Phe), and the other is the isochorismate (IC) pathway that takes place in the chloroplast of the cell with chorismic acid as precursor (Fig. 3). In a broader sense, both pathways actually start from the end product of chikimate pathway – chorismic acid.

For a long period of time, PAL pathway was consider as a major route for SA biosynthesis in plants (Raskin 1992; Coquoz *et al.*, 1998), while IC pathway was consider as alternative found in various microorganisms (Weiss and Edwards, 1980). In the early 1960s it was suggested that in plants SA is synthesized from Phe via *trans*-cinnamic acid by two possible ways: one involves side-chain decarboxylation of *trans*-cinnamic acid to benzoic acid followed by 2-hydroxylation to SA (Fig. 3, route 2b). Alternatively, *trans*-cinnamic acid could be first 2-hydroxylated to an *ortho*-coumaric acid and then decarboxylated to SA (Fig. 3, route 2a). These pathways differ in the order of β-oxidation and ortho-hydroxylation reactions and could operate independently in plants. Third route was detected in tobacco mosaic virus (TMV)-infected tobacco, cucumber and *Nicotiana attenuata* (Ribnicky *et al*. 1998; Jarvis *et al*., 2000) where C, from radiolabelled Phe, was incorporated onto benzoic acid and SA but not benzaldehyde, suggesting that SA is synthesized by the alternative cinnamoyl-CoA β-

oxidation route (Fig. 3, route 2c). This route functions also in rice seedlings (Silverman *et al*., 1995) and healthy tobacco plants (Yalpani *et al*., 1993).

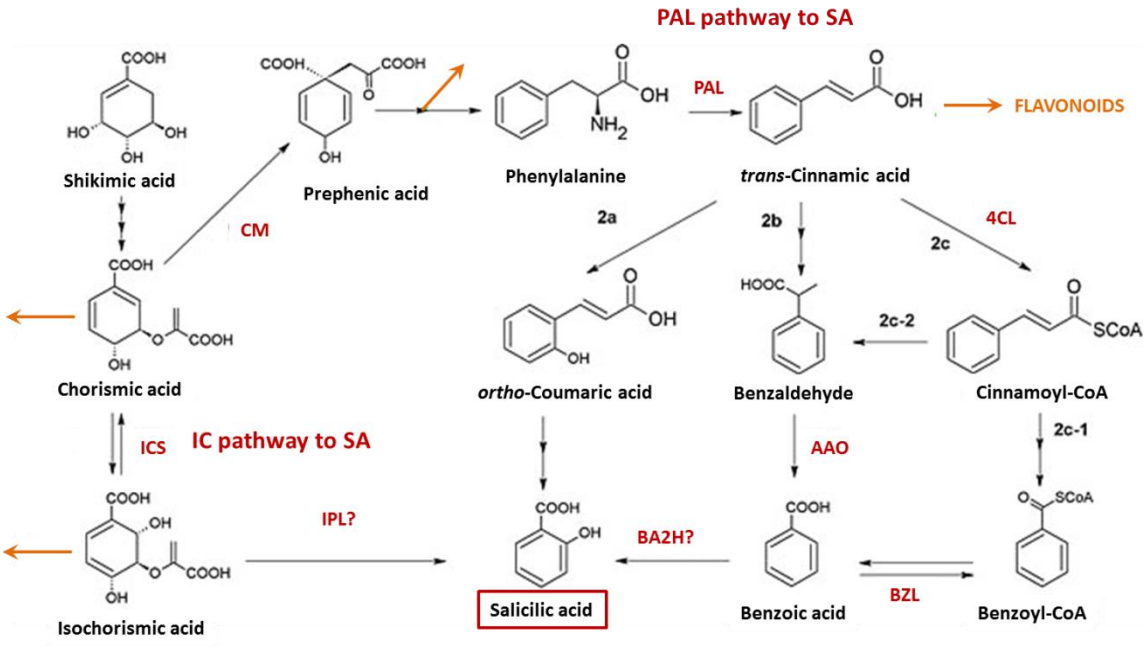

Fig. 3 SA biosynthetic pathways in *Arabidopsis thaliana*. Black arrows indicate enzymatic reactions in SA pathways and orange arrows indicate reactions leading to other pathways competing for the same substrate. Enzymes abbreviations are shown in red: AAO - aldehyde oxidase, BZL - benzoyl-CoA ligase, BA2H - benzoic acid 2-hydroxylase, 4CL - 4-coumaroyl:CoA ligase, CM – chorismate mutase, ICS - isochorismate synthase, IPL - isochorismate pyruvate lyase, PAL - phenylalanine ammonia lyase. Non-identified enzymes, thus far, are marked with a question tag. Adapted from Tounekti *et al*. (2013) and Dempsey *et al*. (2011).

The IC pathway for SA biosynthesis was firstly detected in bacteria. Several bacterial genera produce SA as a compound important for the production of iron-chelating siderophores (Garcion and Métraux, 2006). Investigations on *Arabidopsis thaliana* revealed that SA was produced even when the PAL biosynthetic routes were either inhibited or the specific activity of radiolabeled SA in feeding tests was lower than expected. SA biosynthesis was going on in chloroplasts from chorismate in a two-step process involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Verberne *et al*., 2000; Wildermuth, 2001; Strawn *et al*., 2007). It seems that the

SA synthesized by IC pathway is accountable for providing local response to pathogens and systemic acquired resistance (SAR) in plants (Wildermuth *et al*., 2001) and implicated in several physiological processes including leaf senescence (Abreu and Munné-Bosch, 2008) and modulation of flowering time (Martínez *et al*., 2004). The bulk of pathogen-induced SA is synthesized via this pathway in *Arabidopsis*, *Nicotiana benthamiana*, and tomato (Catinot *et al*., 2008; Uppalapati *et al*., 2007; Wildermuth *et al*., 2001). *Arabidopsis* encodes two ICS enzymes and SA production, as well as pathogen resistance, is severely compromised in mutants lacking functional ICS1, which appears to be responsible for approximately 90% of SA production induced by pathogens or UV light (Garcion *et al*., 2008; Wildermuth *et al* ., 2001). Besides, the IC pathway was found to be the major route for SA synthesis in both basal and induced thermotolerance in *A. thaliana*, although the PAL pathway was also operative (Garcion *et al*. 2008).

1.3.3. Salicylic Acid Modifications

Most of the SA produced and accumulated in plants is glucosylated or methylated (Fig. 4). SA glucoside (SAG, SA 2-*O*-*β*-D-glucoside) is a major conjugate resulting from glucose conjugation at the hydroxyl group of SA. The other is salicyloyl glucose ester (SGE) produced in small amounts by glucose conjugation at the SA carboxyl group. These conjugation reactions are initiated by cytosolic SA glucosyltransferases (SAGT), which are induced by SA application or pathogen attack in *A. thaliana* and *N. tabacum* plants (Song , 2006; Lee and Raskin, 1998). *Arabidopsis* encodes two SAGT enzymes; one preferentially converts SA into SAG, whereas the other forms the less abundant SA derivative, SGE (Dean and Delaney, 2008). SA is likely synthesized in chloroplasts (Garcion *et al*., 2008; Strawn *et al*., 2007; Wildermuth *et al*., 2001), whereas tobacco (Dean *et al*., 2005) and soybean (Dean and Mills, 2004) SAGs are produced in cytosol where SAGT appears to be localized. SAG is actively transported from the cytosol into the vacuole, where it may function as an inactive storage form that can be converted back to SA (Hennig *et al.*, 1993; Dean and Mills, 2004; Dean *et al*., 2005). The mechanism of SAG hydrolysis to SA is vague at present. One of the hypotheses is that SAG may be hydrolyzed by cell wall-associated β-D-glucan glucohydrolases (Hrmova

and Fincher, 2007). This implies SAG transport from vacuole to appoplast for hydrolysis. The other alternative is existence of still unidentified intracellular hydrolase. SAG may function as a slow release storage form of SA that keeps SAR over extended periods of time (Dean and Mills 2004; Dean *et al*. 2005). Another alternative is that the SAG formation is the first step in SA catabolism.

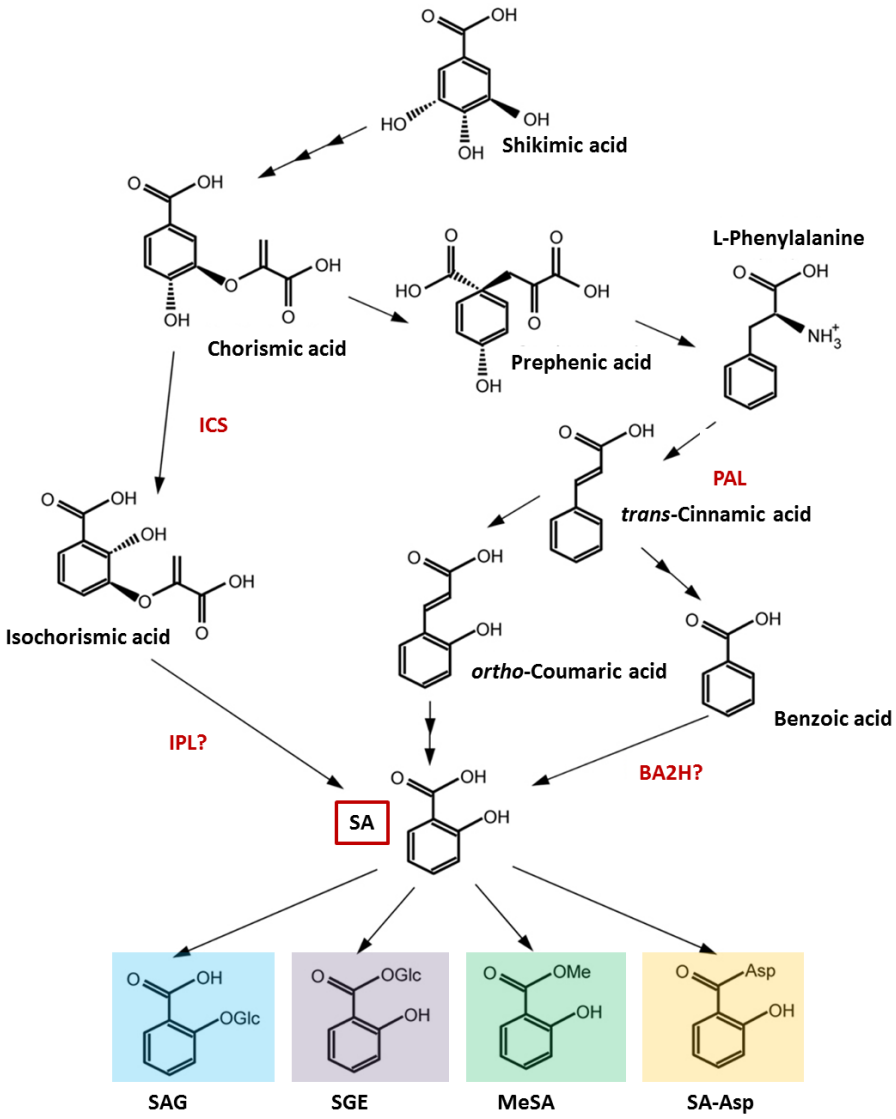

Fig. 4 SA modifications. SAG – salicylic acid 2-*O*-β-glucoside; SGE – salicyloyl glucose ester; MeSA – methyl salicylate; SA-Asp - salicyloyl-L-aspartic acid. Adapted from Miura & Tada (2014).

SA can also be methylated to methyl salicylate (MeSA) by SA carboxyl methyltransferase (Chen *et al*., 2003; Park *et al*., 2007; Vlot *et al*., 2008). Glucoside conjugate SAG in not present in phloem exudates and does not represent translocatable

form of SA. On the other hand, methylation inactivates SA, while increasing its membrane permeability and volatility. MeSA may function as an airborne defense signal, since its release from TMV-infected tobacco or *P. syringae*-infected *Arabidopsis* induced defense gene expression in neighboring plants (Shulaev *et al.*, 1997; Koo *et al*., 2007). In TMV-inoculated tobacco, parallel increase in SA and MeSA occurred in inoculated leaves (Seskar *et al.*, 1998). Interestingly, accumulation of MeSA has been observed also in noninoculated leaves indicating that this compound may function as a signal for both intra- and inter-plant communication in SAR (Seskar *et al.*, 1998; Vlot *et al.*, 2008; 2009). Studies on *Arabidopsis* (Park *et al.*, 2007) and potato (Manosalva *et. al.*, 2010) support this hypothesis. MeSA can be glucosylated to produce MeSA 2-O-*β*-D-glucose (MeSAG), but this SA-conjugated form is not stored in the vacuole (Dean *et al*. 2005). The biological role of MeSAG is unknown.

Amino acid conjugation of SA is less well characterized, but may be involved in SA catabolism (Dempsey *et al.*, 2011). Salicyloyl aspartic acid (SA-Asp) was identified in wild grapes (Silverman *et al*., 1995), *Phaseolus vulgaris* (Bourne et al., 1991) and *Arabidopsis* (Zhang *et al.*, 2007). SA has been shown to be sulfonated *in vitro* by members of the family of sulphotransferases (SOT) in *A. thaliana* (Baek *et al*. 2010), but sulphonated SA has not been detected in planta so far. SA can be potentially converted to the 2,3-dihydroxybenzoate (2,3-DHBA) and 2,5- dihydroxybenzoate (2,5-DHBA). Levels of 2,3-DHBA were increasing in *Arabidopsis* leaves in response to aging, while exogenously applied 2,3-DHBA was a weak inducer of *PR-1* expression as compared with SA (Bartsch *et al.*, 2010), leading to assumption that 2,3-DHBA may represent a de-activated form of SA. SA sulfonation, as well as potential conversion to dihydroxybenzoates, can be part of SA catabolism. However, SA catabolic pathways *in planta* are still unknown.

1.3.4. Roles of Salicylic Acid in Plant Growth and Development

It is well documented that many phenolic compounds play an essential role in the regulation of different physiological processes, including plant growth and development, ion uptake and photosynthesis. SA is one of the plant growth regulators that are involved in many physiological processes in plants. Along with phytohormones,

SA contributes to the hormonal homeostasis in order to preserve the growth rate and defense against biotic and abiotic stress factors, such as pathogens and insects or drought, salinity, high temperature, cold and others (Laloi *et al*., 2004). The importance of salicylic acid in maintaining growth and photosynthesis under unfavorable conditions has been proven.

SA influences a number of processes and stages in plant growth and development:

- flowering
- seed germination
- seedling establishment
- nitrogen metabolism
- nodulation in legumes
- senescence
- thermogenesis in voodoo lili flowers

Effect on some of these processes might be indirect, since SA interacts with phytohormones, i.e. influences the biosynthesis and/or signaling pathways of ethylene (ET), jasmonic acid (JA) and auxins (Yusuf *et al.*, 2013). According to Raskin (1992a), SA is categorized as a plant hormone.

1.3.4.1. Flowering

SA might be one of plant endogenous factors which regulate flowering time. In several plant species, such as gloxinia and *A. thaliana*, earlier flowering was induced by SA-foliar spraying (Martin-Mex *et al.*, 2003; Martínez *et al*., 2004). The significantly higher fruit set, as a result of SA-induced increase in the number of flowers, has been observed in a number of species including cucumber, tomato (Larqué-Saavedra and Martin-Mex, 2007), and *Carica papaya* (Martin-Mex *et al*., 2005a). Flowering was also induced in tobacco *in vitro* culture when plants, grown on medium supplemented with kinetin (Kin) and indole-3-acetic acid (IAA), were treated with SA (Lee and Skoog, 1965). The induction of flowering was also observed in *Lemna paucicostrata* LP6 culture on Bonner-Devirian medium, when 10 μM SA or benzoic acid was added (Khurana and

Cleland, 1992). Handro *et al*. (1997) reported that under short day conditions, addition of SA significantly increased the production of flower buds in *Streptocarpus nobilis* culture *in vitro*. Cleland and Ajami (1974) were analyzing effect of different fractions of honeydew, collected from aphids feeding on flowering and non-flowering *Xanthium strumarium*, on *Lemna gibba* plants. SA has been identified as *X. strumarium* phloem-mobile, active substance competent to induce flowering in *L. gibba*. However, these findings in *Xanthium* and *Lemnaceae* are somewhat questionable. In *X. strumarium*, levels of SA were not different in honeydew collected from vegetative and flowering plants and SA, actually, did not induce flowering. Also, similar endogenous levels of SA were detected in vegetative or flowering *Lemna* and SA induction of flowering *in vitro* was not that specific since benzoic acid have had similar effect.

1.3.4.2. Seed Germination and Seedling Establishment

The SA influence on seed germination and seedling establishment was mainly examined by exogenous application of the compound. Shakirova (2007) reported enhanced seed germination and seedling growth in wheat when grains were soaked in SA solution prior sowing. Also, the treatment of wheat plants with 0.05 mM SA increased the level of cell division within the apical meristem of seedling roots causing an increase in plant growth and better productivity (Shakirova *et al.*, 2003). The simulative effect on plant growth (leaf number, fresh and dry mass per plant) has been also observed in wheat plants raised from grains treated with 0.01 mM SA prior sowing (Hayat *et al.*, 2005). Similar effect has been generated in barley seedlings sprayed with SA solution (Pancheva *et al*., 1996). Conversely, SA concentrations higher than 1 mM and 0.25 mM were inhibiting germination in *A. thaliana* (Rajjou *et al.*, 2006) and barley (Xie *et al.*, 2007), respectively. In maize, seed germination was completely inhibited when SA concentrations of 3-5 mM were applied (Guan and Scandalios, 1995). These contradictory effects can be explained by SA concentrations used in studies. The negative effects of relatively high SA concentrations may be due to SA-induced oxidative stress. When *Arabidopsis* plants were treated with 1-5 mM SA, significant increase in the level of H_2O_2 has been observed as a result of increased activity of H_2O_2-producing Cu/ZnSOD and inactivation of H_2O_2-degrading catalase and ascorbate

peroxidase (Rao *et al.*, 1997). Although mechanisms underlying SA-stimulation of seed germination and plant growth are still not determined, some reports suggest that SA might be involved in regulation of the cell cycle (Carswell *et al.*, 1989) or cell expansion (Miura *et al.*, 2010). In *Arabidopsis*, two xyloglucan endotransglucosylase/hydrolase (*XTH*) involved in cell wall loosening and expansion were found to be SA-regulated (Miura *et al.*, 2010).

1.3.4.3. Nitrogen Metabolism and Nodulation in Legumes

The SA affects nitrate/nitrogen metabolism in plants by modulating activity of enzymes involved, such as nitrate reductase (NR), or legume-Rhizobium symbiosis. In wheat, the NR activity, as well as protection of this enzyme from the action of proteinases, was enhanced following SA application (Rane *et al.*, 1995). Kumar *et al.* (1999) reported increase in NR activity and total protein content in soybean plants treated with SA. Lower SA concentrations (10^{-5} M) induced significant increase in NR activity in wheat (Hayat *et al.*, 2005) and mustard (Fariduddin *et al.*, 2003). Higher SA concentrations (10^{-4} and 10^{-3} M), however, reduced activity of NR. Suppression of SA accumulation plays important role during early stages of Rhizobium-legume symbiosis. During plant-pathogenic microbe interactions, there is increase in endogenous SA which leads to acquired resistance to subsequent infections, i.e. SAR. Compatible rhizobia, however, produce Nod factors in response to legume produced flavonoids and these Nod factors are detected by host legume to suppress SA content during the early stages of nodulation (Mabood and Smith, 2007). Blilou *et al.* (1999) reported that amount of SA remained at basal level or declined when wild-type pea (*Pisum sativum*) plants were inoculated with compatible *Rhizobium leguminosarum*, while increase in SA content was detected when plants were inoculated with *R. leguminosarum* NodC mutant defective in Nod factor biosynthesis. Nonetheless, exogenous application of SA ($\geq 10^{-4}$ M) inhibits growth of rhizobia and, consequently, delays nodule formation and decreases the number of nodules at the roots of plant, which was observed in several legumes, including *Vicia sativa* ssp. *nigra*, pea, alfalfa and white clover (van Spronsen *et al.*, 2003). Similar effects regarding nodule primordia formation and a reduction of the number of emerging nodules have been observed in alfalfa (*Medicago sativa*) plants

inoculated with *Rhizobium meliloti* after 2.5 x 10^{-5} M SA treatment (Martinez-Abarca *et al.*, 1998).

1.3.4.4. Senescence

Senescence is developmentally regulated process in plants which allows recycling of resources from old, senescent leaves into young leaves and/or seeds, and storage organs. There are number of findings which suggest that accumulation of SA is accountable for chlorophyll degradation (yellowing) and increased reactive oxygen species (ROS) levels (necrosis) in senescent leaves. Significant, four-fold increase in SA accumulation has been detected in *Arabidopsis* mid-senescent leaves (Yusuf *et al.*, 2013). Delayed yellowing and lessened necrosis has been observed in *Arabidopsis* impaired in SA-accumulation, *nahG* transgenic plants, or disturbed SA signaling, *npr1*and *pad4* mutants (Morris *et al.*, 2000). Expression of SA-inducible SAG12 cysteine protease, closely linked to senescence and chlorosis, was significantly reduced in *npr1*and *pad4* mutants, and undetectable in *nahG* transgenes. Besides *SAG12*, SA induces expression of senescence-related genes that encode a protease, *SEN1*, two transcription factors, *WRKY6*, and *WRKY53*, and two vacuolar processing enzymes, *aVPE* and *vVPE* (Schenk *et al.*, 2005; Robatzek and Somssich, 2001; Miao *et al.*, 2004). Microarray analysis of *Arabidopsis* plants revealed that ~ 20% of up-regulated genes in senescent leaves of wild-type plants had at least 2-fold reduced expression in leaves of *nahG* transgenic plants (Buchanan-Wollaston *et al.*, 2005). Although SA role in senescence is evident, possible cross-talk with senescence-promoting (ABA, JA, an ET) or senescence-delaying (CKs and GAs) phytohormones has to be examined.

1.3.4.5. Thermogenesis

SA initiates a remarkable increase in the metabolic heat in the thermogenic flowers of voodoo lily, *Sauromatum guttanum* Schott (Raskin *et al.*, 1987; 1989). It was determined that a ~100-fold increase in SA precedes the onset of thermogenesis in the spadix of inflorescence. Exogenous application of SA also induced thermogenesis, as well as 2,6-dihydroxybenzoic acid and ASA of 33 SA-analogs tested, implying that this effect was very specific (Raskin *et al.*, 1989). SA induces thermogenesis by increasing

the activity of alternative respiratory pathway in mitochondria, which is known for generating heat from the unused potential energy of electron flow. Interestingly, exogenous SA also induces alternative oxidase expression and increased alternative respiration in nonthermogenic plant species, such as tobacco (Van Der Straeten *et al*., 1995; Norman *et al*., 2004) and *Arabidopsis* (Clifton *et al.*, 2005). It is important to mention that the discovery of the regulatory role played by endogenous SA in the thermogenesis and flowering of voodoo lily was the first authentication of SA as a plant hormone (Yusuf *et al.*, 2013).

1.3.5. Effects of SA on Photosynthesis and Stomatal Conductance

SA is a regulator of photosynthesis. Depending on concentration and plant species, SA affects chlorophyll and carotenoid content (Chandra and Bhatt, 1998; Fariduddin *et al.*, 2003), leaf and chloroplast structure (Uzunova and Popova, 2000), stomatal closure (Waseem *et al.*, 2006; Saruhan *et al.*, 2012) and activity of photosynthetic enzymes (Pancheva *et al.*, 1996; 1998; Slaymaker *et al.*, 2002; Hayat *et al*., 2012).

Foliar application of SA was found to increase the chlorophyll content in cowpea (Chandra and Bhatt, 1998), *Brassica napus* (Ghai *et al.*, 2002), *Brassica juncea* (Fariduddin *et al.*, 2003) and tomato crops (Kalarani *et al.*, 2002). Soaking the grains of wheat in 10^{-5} M solution of SA resulted in higher pigment contents in the plants, which declined with the increase in SA concentration above 10^{-5} M (Hayat *et al.*, 2005). Salicylic acid treatments stimulated photosynthetic machinery, increased the content of chlorophyll, as well as blocked wound response in soybeans (Zhao *et al*., 1995). However, there are also findings about reduction of chlorophyll content in barley and black gram, *Vigna mungo*, plants treated with SA (Pancheva *et al.*, 1996; Anandhi and Ramanujam, 1997). SA induced biosynthesis of carotenoids and xanthophylls in wheat and mung bean seedlings, but also caused increase in the rate of de-epoxication and decline in chlorophyll pigments (Moharekar *et al.*, 2003).

Stomatal behavior and regulation are a very important factor for photosynthetic proficiency. Larqué-Saavedra (1978, 1979) reported that applied ASA significantly reduced transpiration rate in *Phaseolus vulgaris* and *Commelina communis*. The mechanism of action of ASA in this physiological response is difficult to explain. It was

suggested that ASA might be affecting CO_2 levels within the leaf, thereby inducing stomatal closure (Rai *et al*., 1986). Closure of the stomatal aperture prevents water loss, but it also limits the uptake of carbon dioxide and decreases photosynthetic rate, which exerts a harmful effect on the growth and productivity of plants (Pinheiro and Chaves 2011). Moreover, the limitation of CO_2 assimilation may induce the accumulation of ROS and H_2O_2, because the reductive power (NADPH) developed in the light phase of photosynthesis is not utilized (Jaspers and Kangasjärvi 2010; Miller *et al.*, 2010; Gill and Tuteja, 2010). ROS might have additional regulatory functions in defense mechanisms in conjunction with other plant growth regulators, such as SA, ABA and JA. However, an increase in transpiration rate and stomatal conductance has been observed after SA application in corn and soybean (Khan *et al.*, 2003; Kumar *et al.*, 2000). Also, the decrease of stomatal conductance under drought was lower in SA-treated (applied to roots/leaves) than untreated plants (Waseem *et al.*, 2006; Saruhan *et al.*, 2012).

SA might be involved in the regulation of some photosynthetic reactions by modulating abundance and/or activity of photosynthetic enzymes. Pancheva *et al.* (1996) demonstrated that long-term, 7-day-treatment of barley seedlings with SA decreased slope of the curve showing the net photosynthetic rate vs. intracellular CO_2 concentration. An explanation for these changes could in part be due to stomatal closure and reduced supply of CO_2. However, these values were not declined in SA-treated plants. This implies that the reduction in photosynthesis probably was a non-stomatal. Indeed, activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, RuBP carboxylase) declined with increase in SA concentration, while the activity of PEP carboxylase increased. It was suggested that one very possible reason for the observed inhibition of photosynthetic ability and Rubisco activity could be the effect of SA on protein synthesis, including Rubisco synthesis. It was found that treatment of barley plants with SA caused a decrease in the level of total soluble protein, in particular in the level of Rubisco. In the presence of 1 mM SA, the level of total soluble protein was about 68% of the control, whereas the level of Rubisco was at about 50% of the control value. The percentage of inhibition on the small subunit was higher, as a result of which the small/large subunit ratio was lower for the experimental variants (Pancheva and Popova, 1998).

1.3.6. The Role of SA in Biotic Stress

SA plays an important role in signaling disease resistance to viral, bacterial, and fungal pathogens in many plant species. The significance of SA in plant resistance against pathogens was first implied by White (1979), who discovered that injection of tobacco leaves with SA, ASA or benzoic acid promotes resistance to TMV by reduction in lesion number (>90%). This was supported by latter findings considering accumulation of SA in the leaves of TMV-resistant *Nicotiana tabaccum* cultivar upon TMV inoculation (Malamy *et al.*, 1990), as well as significant increase in the endogenous SA levels in the phloem sap of *Cucumis sativus* plants infected with tobacco necrosis virus, *Colletotrichum lagenarium* or *Pseudomonas syringae* (Metraux *et al.*, 1990; Smith *et al.*, 1991).

SA is essential for development of systemic acquired resistance (SAR), which can be defined as "SA-dependent enhanced defensive capacity of the entire plant, acquired after a primary, local pathogen infection" (Pieterse *et al.*, 2012). In other words, SAR represents induced, broad spectrum immune mechanism which includes generation of mobile signals, accumulation of the SA and production of the antimicrobial pathogenesis-related (PR) proteins. Upon pathogen infection, SA synthesis is triggered after recognition of pathogen associated molecular patterns (PAMPs) or effectors of pathogens, mainly by IC pathway (Vlot *et al.*, 2009). Based on rise in SA level with or just prior to SAR, SA detection in the phloem of pathogen-infected plants (Metraux *et al.*, 1990; Rasmussen *et al.*, 1991; Yalpani *et al.*, 1991) and radio-tracer studies that tracked labeled SA from the inoculated leaf (Mölders *et al.*, 1996; Shulaev *et al.*, 1995), it was proposed that SA represents a signal molecule generated in the inoculated leaf transported via the phloem to the uninfected parts of the plant. However, there are results that question SA role as a SAR long-distance signal. In grafted tobacco plants, SA-deficient (*nahG*-expressing or PAL-suppressed) rootstock leaves generated a signal after TMV infection that activated SAR and systemic *PR* expression in wild type scion leaves (Vernooij *et al.*, 1994; Pallas *et al.*, 1996). Also, results of leaf detachment assays implies that the mobile SAR signal moved out of the infected leaf before detection of increased SA levels in petiole exudates from that leaf (Rasmussen *et al.*, 1991; Smith *et al.*, 1991). Currently, MeSA has been proposed as a

long-distance signal, based on importance of SABP2's methyl salicylate esterase activity in the non-inoculated tissues for SAR development (Park *et al.*, 2007; Vlot *et al.*, 2009). According to Park *et al.* (2007), the locally synthesized SA is first converted to MeSA through the activity of SA methyltransferase and then mobilized to the systemic tissue, where it is converted back to SA by SABP2.

Recent findings indicate that SA controls stability of the *Arabidopsis* nonexpresser of *PR* genes 1 protein (NPR1) (Fu and Dong, 2013). NPR1 is a master regulator of SAR. SA directly binds to the adaptor proteins NPR3 and NPR4 and regulates their interactions with NPR1, which affects NPR1 protein stability (Fu Z. *et al.*, 2012). Besides, SA induces fluctuation in cellular redox, which may cause monomerization of NPR1 (Fu and Dong, 2013). In SA-induced cells, large amounts of monomeric NPR1 translocate to the nucleus and interact with members of the TGA subclass of the basic leucine zipper (bZIP) family of transcription factors. These transcription factors then bind to the promoters of SA-responsive genes, such as *PR* genes, resulting in their activation (Després *et al.*, 2000; Fan and Dong, 2002). PR proteins are small (5–75-kDa) secreted or vacuole-targeted proteins with antimicrobial activities, such as β-1,3-glucanase which degrades glucans found in fungal cell wails (encoded by *PR2* gene) (Fu and Dong, 2013).

1.3.7. The Role of SA in Abiotic Stress Tolerance

Plants are challenged by a variety of abiotic stresses. Several plant growth regulators, including jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and probably hydrogen peroxide (H_2O_2) orchestrate the induction of defenses (Reymond and Farmer, 1998). Salicylic acid (SA) is one of the key factors determining the fate of plants exposed to such stressful conditions. SA has attracted considerable scientific interest because of its ability to induce thermotolerance in mustard seedlings (Dat *et al.*, 1998 a, b), potato (Lopez-Delgado *et al.*, 2004), tomato (Senaratna *et al.*, 2000), and *Arabidopsis* (Larkindale and Knight, 2002; Clarke *et al.*, 2004). Recent studies also describe potentially valuable effects of salicylate treatment on cold tolerance in maize (Janda *et al.*, 1999), rice (Kang *et al.*, 2002), wheat (Taşgín *et al.*, 2003), bean (Senaratna *et al.*, 2000), cucumber (Kang *et al.*, 2002), tomato (Senaratna *et al.*, 2000; Ding *et al.*, 2002)

and Persian lilac (Bernard *et al.*, 2002). Data have been obtained concerning the SA induced increase in the resistance of wheat seedlings to salinity (Shakirova and Bezrukova, 1997; Shakirova *et al*., 2003). Also, SA has ability to induce a protective response in plants exposed to several abiotic stress factors simultaneously – multiple stresses (Senaratna *et al.*, 2000).

The ability of SA to induce plant tolerance may be explained by promotion of ROS formation in the photosynthetic tissues of plants during salt stress, osmotic stress (Borsani *et al.*, 2001) and water deficit (Bezrukova *et al.*, 2001), low and high temperature (Senaratna *et al.*, 2000) or due to the increased activation of aldose reductase and ascorbate peroxidase (APX) enzymes and the accumulation of osmolytes, such as sugars, sugar alcohols or proline (Tari *et al.*, 2002, 2004; Szepesi *et al.*, 2005). Recently it was shown that a PIP_2-specific-phospholipase induction by heat acclimation was mediated by increased levels of free SA (Liu *et al.*, 2006 a, b). SA also has the ability to enhance the heat shock response (HSR) by potentiating the heat-induced levels of heat shock proteins (HSP) in plants (Cronjé and Bornman, 1999; Cronjé *et al.*, 2004). SA potentiated the basal levels of heat-shock factor A1 (HSFA1) in tomato seedlings at room temperature (Snyman and Cronje, 2008). In heat-shocked plants, SA enhanced the binding of HSFs to DNA, gene expression of HSFA1, HSFA2 and HSFB1, as well as gene expression of HSP70.

1.4. Heat Stress

Heat stress (HS) can be defined as metabolic, physiological and morphological changes (damage) induced by elevated temperature in living organisms, including plants. The HS-temperature threshold is often defined as a value of daily mean temperature at which a detectable reduction in plant growth begins, usually being 10-15 ºC above optimal temperature (T). Temperature increase above this threshold may cause irreversible damages to plant function and development or alteration of metabolism, resulting in reduction in growth and yield. Heat stress is a major factor limiting the productivity and adaptation of crops, especially when temperature extremes coincide with critical stages of plant development. At the whole plant level, the effects caused by heat stress include an acceleration of developmental stages resulting in fewer and

smaller organs, abortion of seed development and reduced grain filling (Stone, 2001; Porter, 2005). Physiological processes occurring in plants, such as photosynthesis, respiration and assimilate partitioning are directly affected by high ambient temperature (Wahid *et al.*, 2007a).

Active growth in mesophytes occurs between 10-40 °C. Any temperature which is above or below that range creates perturbation of metabolic activities. Above that range, reaction rates decrease since enzymes are step by step denatured or inactivated. Reactions catalyzed by enzymes depend on completeness of quaternary and tertiary structure of enzymes. Frequent molecular collisions at high temperatures, harms enzyme structure and, therefore, reduce enzyme activity. Also, increase in temperature can cause complete protein denaturation and aggregation. When an enzyme is inactivated, processes related to that enzyme are interrupted. Perturbation of cellular metabolic processes, consequently, affects plant growth and development.

The membrane stability and its functions are also susceptible to high temperature (Wahid *et al*., 2007b). Sudden heat stress may change activities of membrane located transport proteins and receptors, denature membrane proteins, increase the fluidity of the lipid bilayer and induce formation of hexagonal II phase, leading to membrane rupture and loss of cellular contents (Levitt, 1980; Fu J. *et al*., 2012). Due to disturbance of electron-transfer chains in chloroplasts and mitochondria, high temperature can also increase production of ROS and cause oxidative stress, leading to lipid peroxidation (Bhattacharjee and Mukherjee, 1998; Liu and Huang, 2000). Lipid peroxidation disrupts bilayer structure of the membranes and increases membrane ion permeability (Sun *et al*., 2000). ROS also may react with the pigments, enzymes and nucleic acids, and modify their structure and function (Møller *et al.*, 2007).

Increased ambient temperature affects plant productivity by damaging photosynthesis (Al-Khatib and Paulsen, 1990). Photochemical reactions in thylakoid lamellae in the chloroplast stroma have been suggested as the primary sites of injury at high temperature (Wise *et al*., 2004). Heat stress may lead to the dissociation of the oxygen evolving complex (OEC), resulting in an imbalance during the electron flow from OEC toward the acceptor side of photosystem II (PSII) (De Ronde *et al*., 2004).

Heat stress may also impair other parts of the reaction center, e.g. the D1 and/or the D2 proteins (De Las Rivas and Barber, 1997). Rubisco activase (RCA) is heat-labile chloroplast protein that regulates activity of Rubisco enzyme. RCA thermal degradation significantly decrease Rubisco activity and, therefore, photosynthetic plant productivity (Law and Crafts-Brandner, 1999).

The rate of temperature change, the duration and degree of temperature increase all contribute to the intensity of heat stress. Plants have developed a number of adaptive mechanisms that enable them to alleviate the negative effects of HS (Larkindale *et al*., 2005; Wahid *et al*., 2007a). Plant response to heat stress depends on the thermal acclimation, the duration of the exposure and the growth stage of exposed tissue (Chen *et al*, 1982).

1.5. Plant Heat Tolerance

Plants are sedentary organisms whose survival depends on the ability to withstand exposure to temperatures above the growth-optimum. High temperature tolerance in the absence of acclimation has been termed basal thermotolerance (Lee *et al.*, 1995; Nieto-Sotelo *et al.*, 2002; Hong and Vierling, 2003). Besides, heat tolerance can be induced in plants by prior exposure to moderately high temperatures which enables the plant to cope with subsequent, potentially lethal, heat exposure (Howarth and Ougham, 1993). This phenomenon, termed acquired thermotolerance, probably represents plant response to the gradual increases in temperature in the natural environment.

The protein synthesizing system plays a crucial role in plant acclimation processes. If the growth temperature of plants is increased beyond a particular point, dramatic changes in gene expression and translation occur. It has been hypothesized that specific proteins whose synthesis is induced by stress conditions are critical for the survival in that stress (Schöffl *et al*., 1988; Scandalios, 1990). Alternatively to the normal pattern, a new set of proteins is synthesized from transcribed mRNAs (Munro and Pelham, 1985), denominated as heat shock protein (HSPs) complement. Pretreatments which lead to the acquisition of thermotolerance are also conditions under which HSPs are synthesized (Kimpel and Key, 1985; Kimpel *et al*., 1990; Nagao *et al*.,

1986). During HS these HSPs could interact with other proteins to prevent their thermal aggregation and degradation, and facilitate resemblance of functional structure.

ROS, ROS-mediated signaling, and enzymatic and non-enzymatic ROS scavenging mechanisms have been also implicated in plant responses to heat stress (Kotak *et al*., 2007; Larkindale and Vierling, 2008; Miller *et al*., 2008; Penfield, 2008). ROS are toxic byproducts of aerobic metabolism which accumulates in cells during abiotic stresses (Miller *et al*., 2008; Jaspers and Kangasjarvi, 2010). During heat stress ROS levels increase dramatically, which results in significant damage to plants macromolecules and cell structures, leading to obstruction in plant growth and development (Gill and Tuteja, 2010). Thus, ROS must be rapidly detoxified by various cellular enzymatic or nonenzymatic mechanisms. To manage oxidative damage and simultaneously regulate signaling events, plants have orchestrated an elaborate antioxidant network system (Foyer and Noctor, 2005; Rouhier *et al*., 2008).

During evolution, plants have developed a series of mechanisms to protect the photosynthetic apparatus against damage resulting from heat stress. One of such mechanisms is partition of xanthophylls (the xanthophyll violaxanthin and the products of its enzymatic de-epoxidation, antheraxanthin and zeaxanthin) between the light-harvesting complexes and the lipid phase of the thylakoid membranes (Tardy and Havaux, 1997; Havaux, 1998). Interaction of the xanthophyll molecules and the membrane lipids cause decrease in membrane fluidity and lower susceptibility to lipid peroxidation, leading to an increase in thylakoid membrane thermostability (Havaux, 1998). However, when plants are subjected to more severe stress, these protective mechanisms may be inadequate. Some plant growth regulators, such as brassinosteroids (BRs), abscisic acid (ABA) and ethylene, have been used to induce or enhance these protective mechanisms (Larkindale and Knight, 2002; Krishna, 2003). SA has received much attention due to its role in plant responses to abiotic stresses (Horváth *et al*., 2007), including heat stress.

1.6. Heat-Shock Proteins

All organisms respond to high temperature by synthesizing a group of distinct polypeptides called heat shock proteins (HSPs) (Lindquist and Craig 1988). Heat-shock

proteins have been classified on the basis of their approximate molecular weight, amino acid sequence homologies and functions into five well-characterized families: HSP100 family, HSP90 family, HSP70 family, HSP60 family, and the small HSP family (sHSPs; molecular size ranging from 15 to 30 kDa) (Vierling 1991; Kotak *et al.*, 2007; Gupta *et al.*, 2010). Small HSPs represent the most diverse and abundant HSPs induced by heat stress in plants (Waters *et al.*, 1996).

HSP synthesis is detectable 20-30 minutes after reaching 10-15 °C-higher temperatures than optimum growth temperature (Cooper *et al.*, 1984; Cooper and Ho, 1984). Maximum transcription of HSP genes is usually accomplished in about an hour or two, while HSPs produced during the onset of heat stress may persist in plant cells for as long as 21 h after the stress (Vierling, 1991).

Although HSPs were first characterized and accordingly named because of their induction during heat-shock, elevated expression of many HSPs has been detected in response to other abiotic stresses (Vierling, 1991). Some HSPs (e.g. HSP70 and HSP60) and cellular proteins homologous to HSPs are produced at significant levels in non-stressed cells, or are produced at particular stages of during development (Vierling, 1991). The term HSP cognate (HSC) has been frequently used to identify HSP family members which are expressed at a significant level in the absence of heat stress. However, specificity of HS response lies in the fact that high temperatures induce expression of entire HSP complement (group).

1.6.1. Regulation of HSP Expression

Molecular pathway leading to the expression of genes to synthesize HSP is composed of several mechanisms, such as mechanism of sensing temperature that is connected to the mechanism of signal transfer to HSFs, leading to the HSP-gene expression by HSFs binding to the heat shock element (HSE) in DNA (Fig. 5) (Schöffl *et al.*, 1998; Larkindale *et al.*, 2005). HSFs are located in the cytoplasm in an inactive state. Findings from previous studies suggest that expression of constitutively active plant HSFs only increases basal thermotolerance, but acquired (induced) thermotolerance is unaffected (Lee *et al.*, 1995; Prändl *et al.*, 1998).

Plants are characterized by a large number of transcriptional factors. In *Arabidopsis thaliana*, the response to heat shock treatment occurs through the participation of a number of different HSPs (HSP 21, HSP70, HSP90, HSP100) and at least 21 HSFs (Nover and Baniwal, 2006; Swindell *et al*., 2007). Plant HSFs have been classified (Tripp *et al.*, 2009) into three classes according to the structural differences in oligomerization domains, as follows:

- Plant HSFA such as HSFA1 and HSFA2 in *L. esculentum*
- Plant HSFB such as HSFB1 in *L. esculentum*
- Plant HSFC

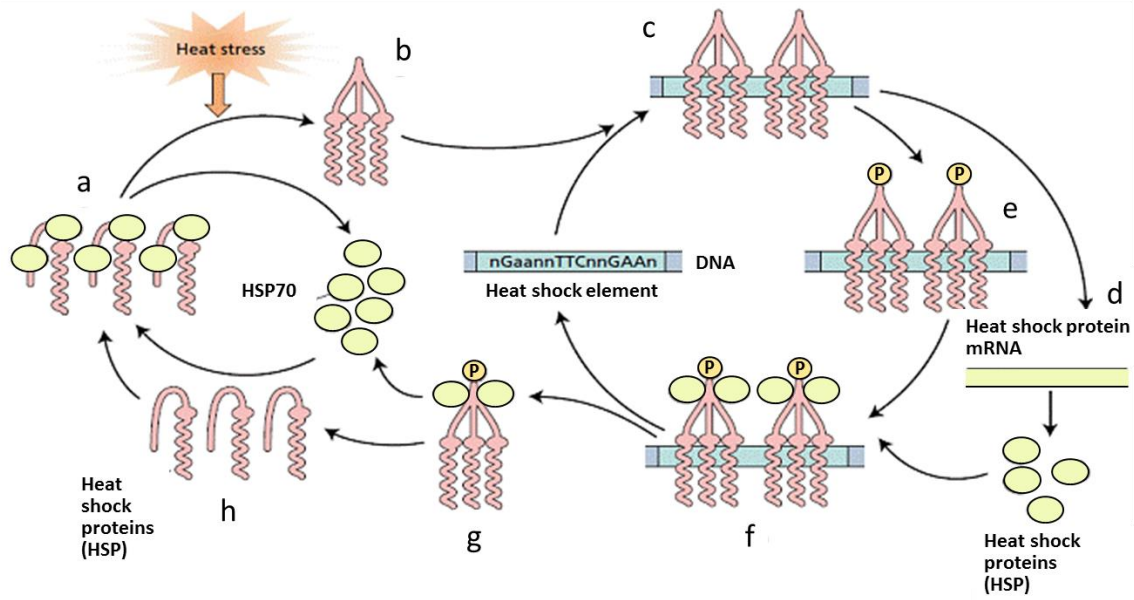

Fig. 5 The role of heat shock factors (HSFs) in the synthesis of heat shock protein mRNAs. In non-stressed cells, HSF exists in a monomeric state associated with HSP70 proteins (a). After high temperature incidence, HSP70 dissociates from HSF which subsequently trimerizes (b). Active trimers bind to heat shock elements (HSE) in the promoter of heat shock protein (HSP) genes (c), and activate the transcription of HSP mRNAs. This leads to the translation of HSPs, including HSP70 (d). The HSF trimers associated with the HSE are phosphorylated (e) promoting the binding of HSP70 to the phosphorylated trimers (f). The HSP70-HSF-trimer complex (g) dissociates from the HSE, disassembles and dephosphorylates into HSF monomers (h). HSF monomers bind HSP, reforming the HSP70/HSF complex. Adapted from Taiz & Zeiger (2006).

Each HSF has its role in the regulatory network in plants, but they all cooperate in regulating many functions and different stages (triggering, maintenance, and recovery) of response to periodical heat stress. In tomato, HSFA1a is the master regulator responsible for the stress-induced gene expression, including the synthesis of other HSFs, HSFB1 and HSFA2 (Mishra *et al.*, 2002). Factor HSFA2 is also finely regulated with HSP17-CII during anther development of a heat-tolerant tomato genotype and was further induced under both short and prolonged heat stress conditions (Giorno *et al.*, 2010). These three factors, HSFA1a, HSFA2 and HSFB1 are necessary for plant acquisition of heat tolerance (Baniwal *et al.*, 2004); however there are at least 15 HSFs in tomato (von Koskull-Doring *et al.*, 2007; Mishra *et al.*, 2002). Interestingly, SA potentiated the basal levels of HSFA1 in tomato seedlings at room temperature (Snyman and Cronje, 2008), while had no influence on the transcription of HSP70 mRNA or the expression of HSFA2 and HSFB1. In heat-shocked plants, however, SA enhanced the binding of HSFs to DNA, gene expression of HSFA1, HSFA2 and HSFB1, as well as gene expression of HSP70. These findings suggest SA role in modulation of the HSFs expression and activity, which may potentiate HSP expression.

1.6.2. Roles of Heat-Shock Proteins

HSPs are molecular chaperones responsible for the establishment of a functional protein conformation, translocation and degradation of proteins in many cellular processes. HSPs also stabilize proteins and membranes and interact with partially denatured proteins in order to restore their native conformation during stressful conditions. (Sun *et al.*, 2002; Basha *et al.*, 2012). Proteins are rarely static. Rather, proteins are continuously accessing multiple conformations that are required for their biological activity. Therefore, even native proteins are susceptible to aggregation during these conformational transitions, which along with environmental variations in temperature or redox status, cause proteins to expose buried hydrophobic surfaces (Englander *et al.*, 2007). The hydrophobic residues of denatured proteins may interact, causing protein aggregation. Protein aggregation resulting from stress and disease represents a major threat to all living cells. A cellular protein quality control network consisting of molecular chaperones and proteases regulates damage caused by protein aggregates

(Liberek *et al.*, 2008). Molecular chaperones interact with the hydrophobic surfaces of unfolded proteins, preventing their aggregation and promoting refolding.

HSP70 chaperone, together with their co-chaperones, DnaJ/HSP40 and GrpE, represent ”chaperone machine” involved in protein folding processes in almost all cellular compartments (Wang *et al.*, 2004). Some members of HSP70 family are constitutively expressed and are often referred to as heat-shock cognate (HSC70). These family members are often involved in assisting the folding of de novo synthesized polypeptides and the import/translocation of precursor proteins. Other family members, expressed only when the organism is exposed to stress conditions, are more involved in promoting refolding and proteolytic degradation of non-native proteins. HSP70 also plays a regulatory role in the expression of other HSPs. The interaction between HSP70 and HSF may represent a negative regulatory mechanism for HSF-mediated transcriptional activation in the HSR (Section 1.7.2.).

HSP60 with co-chaperone HSP10, also known as chaperonin 60 (Cpn60) and co-chaperonin 10 (Cpn10), represents intricate protein-folding complex. The term chaperonin was first suggested to describe a class of molecular chaperones that are evolutionarily homologous to *E. coli* chaperone GroEL and co-chaperone GroES (Hemmingsen *et al.*, 1988). Chaperonins are composed of fourteen 60 kDa monomers that are arranged in the form of a large cylindrical oligomer composed of two stacked heptameric rings (Horwich *et al.*, 2007). A given protein may undergo multiple rounds of binding, encapsulation, and release by HSP60/HSP10 to regain native conformation. Chloroplast chaperonins are important in folding and assembly of plastid proteins such as Rubisco (Wang *et al.*, 2004). Also, stromal HSP60, in cooperation with HSP70, assists newly imported chloroplast proteins to regain their functional conformation (Jackson-Constan *et al.*, 2001). Chloroplast chaperonins comprise two distinct class of isoforms (α and β) which can combine to form oligomeric structures (Vitlin Gruber *et al.*, 2013). The incorporation of unique subunits into the oligomer can modify its substrate specificity, as well as mode of regulation**.** Some subunits genes, such as *Cpn60β2*, are up regulated in response to heat shock (Vitlin Gruber *et al.*, 2013), which may represent way of regulation of HSP60 activity under HS.

HSP90 has important role in promoting protein folding, but it also plays significant role in cell-cycle control, signal-transduction networks, protein trafficking and protein degradation (Young *et al.*, 2001; Richter and Buchner, 2001; Pratt *et al.*, 2001). To perform its functions, HSP90 co-operates with HSP70 and a number of co-chaperones, including Hip, Hop, p23 and HSP40 (Wang *et al*., 2004). Similarly to HSP70 and HSP60, HSP90 requires ATP as a source of energy for large conformational changes underlying its chaperone activity. HSP90 proteins are constitutively expressed in most organisms. However their expression increases in response to stress. In *Arabidopsis*, expression of HSP90 is responsive to heat stress, cold stress, salt stress and heavy metal-induced stress (Milioni *et al.*, 1997; Krishna *et al.*, 2001).

Families of HSP101 and sHSPs are of great importance in plant HS response. Because of the abundance and diversity of sHSPs in plants, extensive studies have focused on the biological functions of plant sHSPs (Sun and MacRae, 2005). It is believed that this diversification and abundance of the sHSPs in plants reflect an adaptation of the plant to heat stress (Waters *et al.*, 1996).

1.6.3. Small HSPs

Higher plants are characterized by the presence of at least 20 types of sHSPs, but some species can contain even 40 types of these proteins (Vierling, 1991; Al-Whaibi, 2011). The number of sHSPs varies among plant species: *Arabidopsis* has 19 sHSPs (Scharf *et al.*, 2001; Waters *et al*., 2008a), rice (*Oryza sativa*) has 23, and poplar (*Populus trichocarpa*) has 36 (Waters *et al*., 2008a).

All plant sHSPs encoded by nuclear genes are divided into a number of subfamilies. Using analysis of the available angiosperm complete genome sequences, researchers have identified 11 sHSP subfamilies including six subfamilies that are cytoplasmic/nuclear localized (CI–CVI) and five sHSP subfamilies that localize to organelles (Scharf *et al.*, 2001; Siddique *et al.*, 2008; Waters *et al.*, 2008a; Sarkar *et al.*, 2009; Bondino *et al.*, 2012). The organelle subfamilies include one subfamily that localizes to the endoplasmic reticulum (ER), another that localizes to the peroxisome (PX), one subfamily that localizes to the chloroplast (CP), and two subfamilies that localize to the mitochondria (MTI and MTII). Ten of these subfamilies are present in

both monocots and dicots (CP, MTI, MTII, ER, PX, CI, CII, CIII, CIV, and CV), and one is found only in dicots (CVI).

The sHSPs are characterized by a core α-crystalline domain of 100 amino acids (ACD), which is flanked by an N-terminal arm of variable length and divergent sequence and a short C-terminal extension (Haslbeck *et al*., 2005). ACD has a typical β-sheet structure. It is composed of eight β-strands (β2−β9) as seen by the X-ray examination of *Methanococcus jannaschii* HSP16.5 and *Triticum aestivum* HSP16.9C-CI (Kim *et al.*, 1998a; van Montfort *et al.*, 2001a). Despite the considerable conservation of ACD across the plant sHSPs, some interesting differences exist among plant sHSP subfamilies. Two plant subfamilies (CIV and CV) lack the crucial β6 sheet. This is notable because it has been shown that the β6 sheet is important for dimer formation (Kim *et al.*, 1998b; van Montfort *et al.*, 2001a) and these sHSPs might have a different overall quaternary structure. However, it has been demonstrated that *Arabidopsis thaliana* HSP18.5 CIV can form dimers despite the lack of the β6 sheet (Siddique *et al.*, 2008). In cells, sHsps typically exist as multimeric complexes of 8 to 24 or more subunits (200 to 800 kD) with dimers as building blocks (Kim *et al.*, 1998a; van Montfort *et al.*, 2001b; Stamler *et al.*, 2005). Recent findings indicate that sHSPs are polydisperse, meaning that they can exist in more than one oligomeric state (Baldwin, 2011).

Protein denaturation due to heat stress is reversible unless followed by aggregation. Small HSPs are first line of defense in the cell when proteins begin to unfold (Waters, 2013). They have a very high capacity for binding denatured substrates (probably by exposing hydrophobic surfaces) and do not require ATP to bind substrate proteins (Nakamoto and Vigh, 2007). According to current models, sHSPs bind substrate proteins in an ATP-independent manner during heat stress, preventing their aggregation and maintaining them in a state competent for subsequent ATP-dependent refolding, which is promoted by other chaperones (Waters, 2013). Lee and Vierling (2000) have shown that the HSP70 system is required for refolding of a sHSP18.1-bound firefly luciferase.

The molecular chaperone activity of tomato HSP17.7, CI subfamily, and HSP17.3, CII subfamily, were confirmed *in vivo*, as shown by their ability to prevent

the thermal inactivation of firefly luciferase in a transgenic *Arabidopsis thaliana* cell culture (Löw *et al.*, 2000). Also, transgenic carrot cells and plants engineered to constitutively express the HSP17.7 CI gene were more thermotolerant than vector control cell lines and plants, while carrot cells and plants that produce antisense HSP17.7 mRNAs were less thermotolerant than vector controls (Malik *et al.*, 1999). This was the first demonstration that manipulating the expression of a single heat shock gene could both increase and decrease thermotolerance in plants.

Heckathorn *et al.* (1998) demonstrated that HSP21, a nuclear-encoded, chloroplast-localized sHSP (CP subfamily), plays an important role in protecting thermolabile PSII against heat stress *in vitro*, implying the molecular mechanism by which HSP21 is involved in cell protection. Although some studies have shown a correlation between the levels of chloroplast sHSPs and PSII thermotolerance *in vivo* (Heckathorn *et al.*, 2002; Wang and Luthe, 2003), direct evidence of their role in the protection of PSII in planta has not been yet provided.

Recent findings indicate that sHSPs may also play an important role in membrane quality control and thereby potentially contribute to the maintenance of membrane integrity under stress conditions (Nakamoto and Vigh, 2007). Among these, HSP17 proteins have dual role: stabilize heat-stressed membranes and bind denatured proteins in the cytosol for subsequent chaperone mediated refolding (Török *et al.*, 2001). Zhong *et al.* (2013) have found that HSP21 might be essential for chloroplast development in *Arabidopsis* under heat stress. It seems that HSP21 is involved in plastid-encoded RNA polymerase (PEP)–dependent transcription by interacting with plastid nucleoid protein pTAC5. HSP21 and pTAC5 were forming a complex that was associated mainly with the PEP complex during transcription initiation, but also during elongation and termination.

1.6.4. HSP100 Family

Members of this family are constitutively expressed, but they are also up regulated by environmental stresses. HSP101 proteins are involved in resolubilization of non-functional protein aggregates and help degradation of irreversibly damaged polypeptides (Glover and Lindquist, 1998; Bösl *et al*., 2006). It was stated that over-

expression of HSP101 in *Arabidopsis* has positive effect on growth during recovery period after HS (Wang *et al*., 2004). Even so, increase in HSP101 transcript and protein production begins instantly on exposure of plants to temperatures 5–10 °C above optimal growth temperature, 30 °C and above in *Arabidopsis*, and increases dramatically in HS plants (Hong *et al*., 2001, 2003; Young *et al*., 2001). Queitsch *et al*. (2000) have shown that alterations in HSP101 expression influence both basal and acquired thermotolerance. The requirement of HSP101 for acquired thermotolerance is demonstrated by exposure of seedlings to the lethal temperature of 45°C for 2 h with and without preconditioning at 38°C. All plants are killed by direct exposure to the severe heat stress. Pretreatment at 38°C, however, enables wild-type and vector-only controls to survive the subsequent severe heat, whereas plants with reduced HSP101 levels are drastically impaired or killed despite pretreatment. This result provides clear evidence that HSP101 expression is essential for induced thermotolerance.

The function of HSP101 is not restricted to acclimation to high temperatures. Members of the family provide housekeeping functions that are essential to chloroplast development. It was recently found that HSP101 homologue in *Arabidopsis* is involved in both plastid differentiation mediating internal thylakoid membrane formation and thermotolerance of chloroplasts during heat stress (Lee *et al*., 2006, 2007; Myouga *et al*., 2006).

2. OBJECTIVES

2. OBJECTIVES

The major aim of this study was to investigate effects of SA on the basal heat tolerance in potato, *Solanum tuberosum* L. Following are the particular objectives of the study**:**

- to establish *in vitro* shoot cultures of selected potato cultivars: 'Arnova', 'Liseta', 'Marabel' and 'Cleopatra',
- to develop model system for investigation of heat stress in potato grown *in vitro* and to determine relative heat tolerance of investigated potato cultivars,
- to investigate effects of SA pretreatment and treatment on morphology of potato plants exposed to heat stress *in vitro,*
- to investigate effects of SA on the expression of heat-shock proteins, HSP17.6, HSP21 and HSP101, and Cu/ZnSOD in potato plants exposed to heat stress *in vitro*.

3. MATERIAL AND METHODS

3. MATERIAL AND METHODS

This study was carried out on selected commercial varieties of potato in the period of 2010 - 2014 in the Plant Tissue Culture Laboratory and greenhouse of the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade.

3.1. Plant Material

Virus-free tubers of commercial potato (*Solanum tuberosum* L.) cultivars, 'Agria', 'Arnova', 'Liseta', 'Laura', 'Marabel' and 'Cleopatra', were obtained from Solanum Komerc (Guča, Serbia). Tubers were kept in the cold room at 8 ºC in the darkness for two months in order to mature. After that period, tubers were washed with tap water for 30 min., left to dry and stored at 18-25 ºC in darkness in order to induce sprouting. After 2-3 weeks, the sprouts of approximately 1-3 cm were formed.

3.1.1. Greenhouse-Grown Plants

Sprouted tubers were planted in pots (four tubers per pot, one pot per cultivar) containing commercial potting soil (Biohum, Belgrade, Serbia) - vermiculite mixture (3/1= w/w) and grown in greenhouse (average daily temperature: 23 ± 4 °C).

3.1.2. Potato *In Vitro* Culture

Four potato cultivars, 'Arnova', 'Liseta', 'Marabel' and 'Cleopatra', were used for *in vitro* studies. Potato sprouts (1-3 cm) were separated from tubers, first rinsed with fresh tap water 15 min. and then with 70% water solution of ethanol with addition of 3 drops of detergent 5 min. Sprouts were surface sterilized with 20% sodium hypochlorite for 5 minutes with constant stirring. After sterilization, sprouts where rinsed five times with sterile, deionized water. Apical segments of sprouts were transferred on the culture medium.

3.1.2.1. Culture Media and Growth Conditions

For the growth of primary explants (potato sprouts), basal culture medium (Table 1.) composed of MS macro-mineral salts and micro-mineral salts (Murashige and Skoog, 1962), LS vitamins (Linsmaier and Skoog, 1965), 0.7 % agar, 3 % sucrose, 100 mg l^{-1} myo-inositol and supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BA; Sigma Aldrich, St. Louis, MO) has been used. BA was added to promote growth of primary explants, as well as bud and shoot development. Shoots obtained on this medium gave rise to microplants when transferred on the same medium (basal medium; BM) without BA. Microplants were grown in culture jars (250 ml) containing ca 50 ml of medium and closed with polycarbonate caps. They were routinely subcultured every 30 days using single-node stem cuttings (SNC) as explants. The *in vitro* shoot cultures were grown in a controlled environment room at 23 ± 2 °C, 16 h photoperiod and light flux of 45.5 μmol m^{-2} s^{-1}. These cultures were further used as a starting material for experiments.

Table 1. Composition of basal medium (BM) used for potato *in vitro* propagation. Quantities of components per one liter of medium are listed.

MS macro-mineral salts (mg l^{-1})		MS micro-mineral salts (mg l^{-1})		LS vitamins (mg l^{-1})	
NH_4NO_3	165	$MnSO_4x4H_2O$	22.3	Vitamin B1	0.4
KNO_3	190	$ZnSO_4x7H_2O$	8.6	Vitamin B6	0.5
$CaCl_2x2H_2O$	440	H_3BO_3	6.2	Nicotinic acid	0.5
$MgSO_4x7H_2O$	370	KJ	0.83	Glycine	2.0
KH_2PO_4	170	NaMoO4x2H2	0.25	Organic compounds (g l^{-1})	
		$CuSO_4x5H_2O$	0.025		
		$CoClx6H_2O$	0.025	Sucrose	30.0
		$FeSO_4x7H_2O$	27.8	Myo-inositol	0.1
		Na_2EDTA	37.3	Agar	7.0

3.2. Electrolyte Leakage Assay (ELA)

The relative heat-tolerance of cultivars was determined using the electrolyte leakage assay of Savić *et al.* (2012). Ten leaf discs (5 mm in diameter) from 2-month old greenhouse-grown plants were taken, avoiding the leaf veins, placed in glass vials containing 10 ml deionized water and incubated in a water bath with continuous shaking at 23 °C (control) or 50 °C (high temperature treatment). Solution conductivity was measured after 4 h of incubation using an ECScan conductivity meter (Lovibond, Germany). To calculate the total ion leakage caused by maximum plasma membrane damage, the samples were boiled for 15 min at 100 °C and cooled to room temperature prior to measurement of solution conductivity. Electrolyte leakage of samples after 4 h of incubation at 23 °C or 50 °C was calculated as a percent of electrolyte leakage after boiling (R23 °C or R50 °C). The relative cellular membrane damage (CMD) caused by heat treatment was calculated for each cultivar according to following equation:

$$CMD\ (\%) = (R50\ °C - R23\ °C) / (100 - R23\ °C)$$

Two replicates for each cultivar were performed and the entire procedure was repeated 4 times.

3.3. Effects of SA on the Morphology of Potato Plants under Various Temperature Treatments – Experimental Design

Single-node stem cuttings (SNC) from potato microplants grown *in vitro* were transferred on BM supplemented with different concentrations of SA: 10^{-6}, 10^{-5} and 10^{-4} M or without SA (control) (Fig. 6).

After 21 days, developed plantlets were cut into SNC that were transferred either to BM supplemented with the same SA concentrations that was added previously to the medium for growth of plants from which SNC originated (SA treatment) or to BM without SA (SA pretreatment) (Fig. 6). After one day of recovery, following cutting, SNC were subjected to regular growth temperature (23 °C, 20 days), moderately elevated temperature (35 °C, 20 days; long-term HS) or excessively elevated temperature (45 °C, 6 h; short-term HS).

Percent of developed plantlets from SNC, as well as plant morphometric parameters (shoot length, number of internodes, number of roots and root length) were determined immediately after 23 °C- and 35 °C-temperature treatment, or after 20 day-long recovery period following 6 h exposure to 45 °C. All experiments were repeated twice with 20-24 SNC per SA treatment or control.

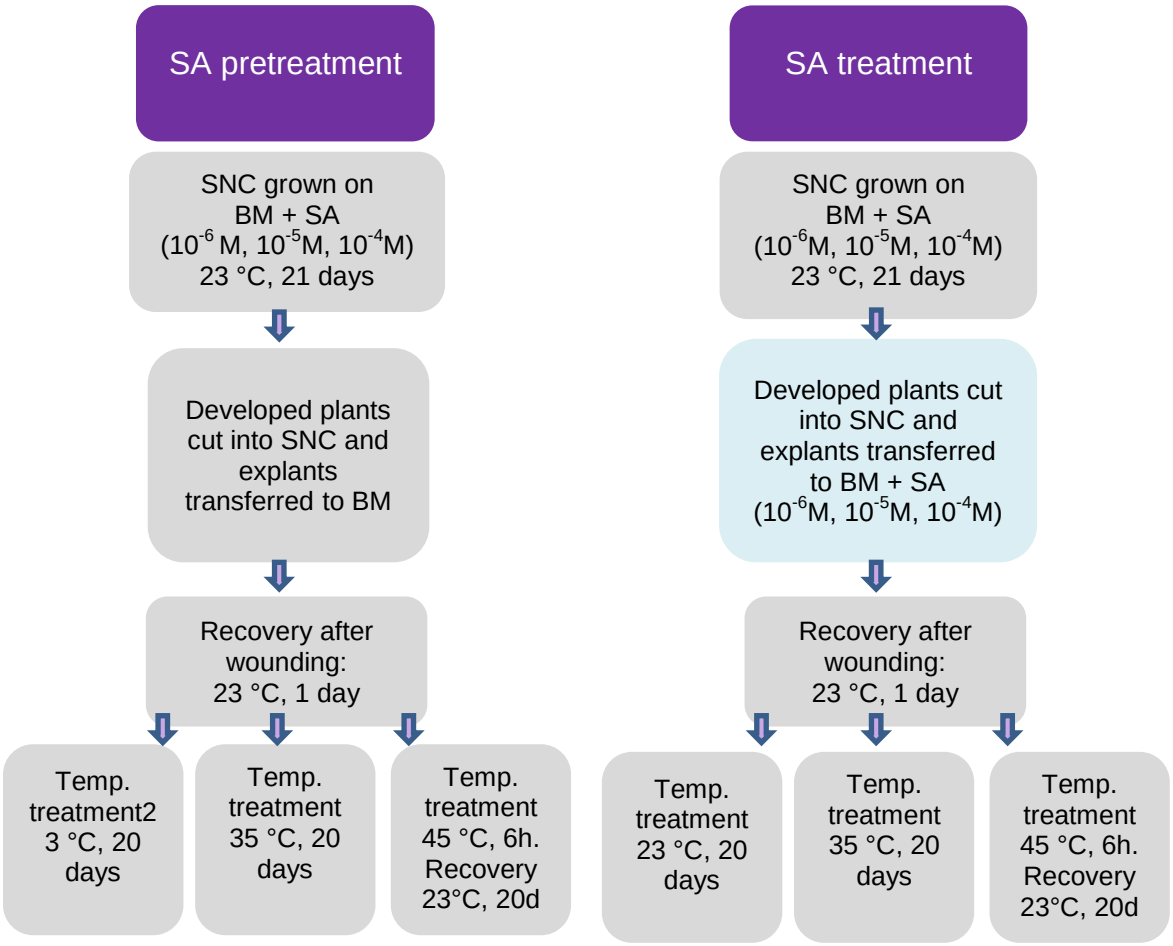

Fig. 6 Design of experiments conducted to investigate effects of SA on the morphology of potato plants.

3.4. Effects of SA on the Expression of HSP in Potato Plants under Various Temperature Treatments – Experimental Design

Single-node stem cuttings (SNC) of potato microplants were transferred on BM supplemented with different concentrations of SA (10^{-6}, 10^{-5} or 10^{-4} M) or without SA (control) (Fig. 7.). Shoots developed after 21 days were transferred to BM without SA. After one day of recovery, following the wounding caused by cutting, shoots were subjected to following temperature treatments: (a) 23 °C (b) 35 °C or (c) 45 °C for 6 h.

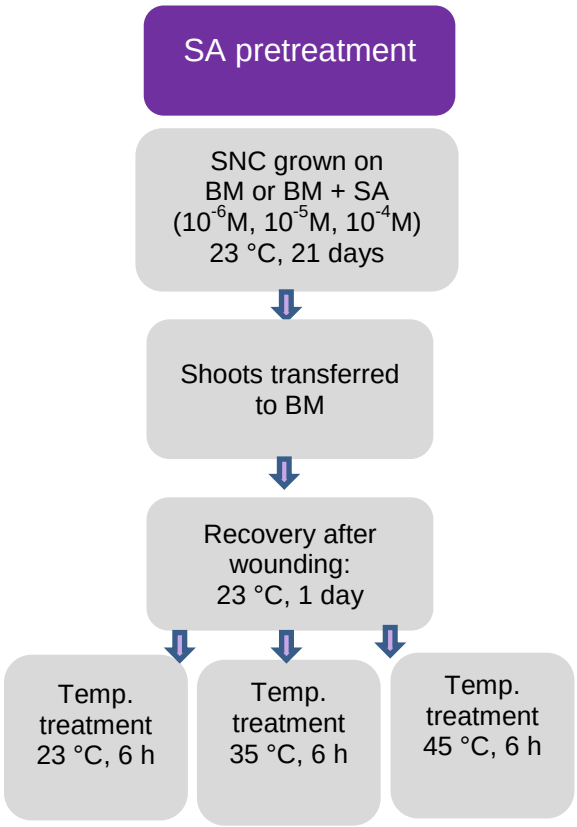

Fig. 7 Design of experiments conducted to investigate effects of SA on HSP expression.

Shoots were collected immediately after temperature treatments, frozen in liquid nitrogen, stored at -70 °C, and used further for protein analysis.

To examine HSP 17.6 expression at different time points after cessation of SA treatment, potato SNC were grown on BM or supplemented with 10^{-5} M SA. Control

plants were grown on BM. After 21 days, shoots were transferred on BM medium without SA. Shoots were exposed to the temperature of 45 °C for 6 h at different time points after transfer: 0, 1, 3, 7, 10 or 14 days (Fig. 8).

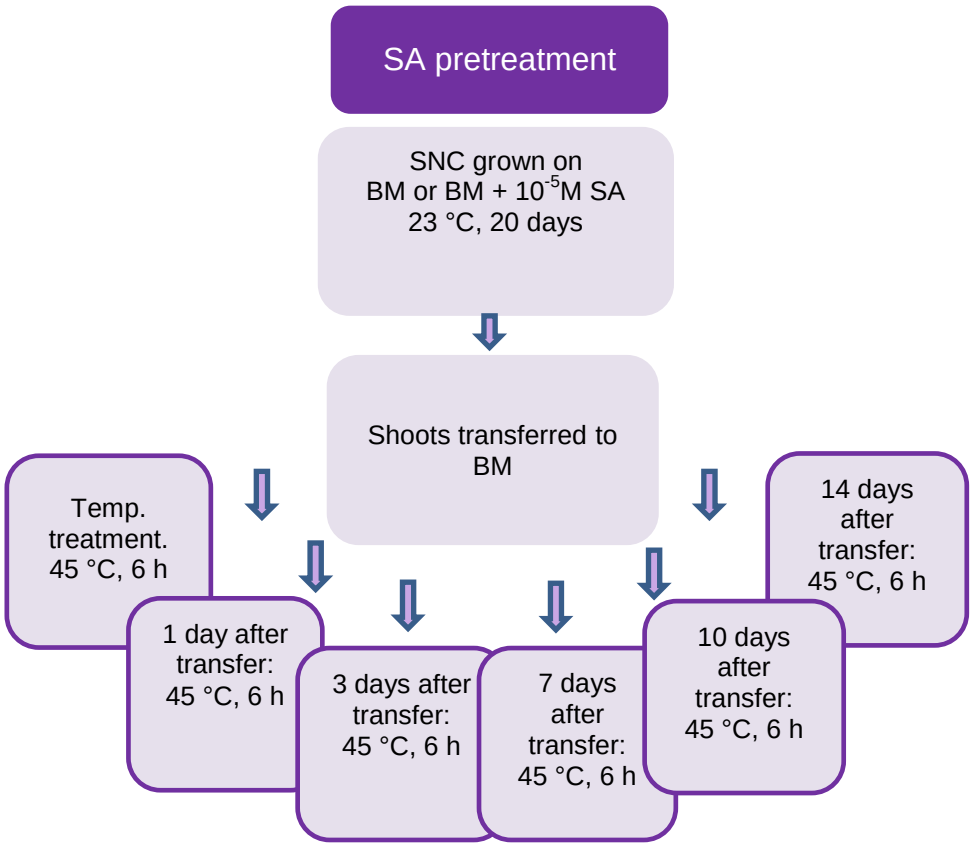

Fig. 8 Design of experiments conducted to investigate effects of SA on HSP 17.6 expression under HS at different time points after cessation of pretreatment.

3.4.1. Isolation of Soluble Leaf Proteins

Leaves were grinded in a mortar to a fine powder after adding liquid nitrogen. Total soluble leaf proteins were isolated by addition of extraction puffer [2 mM EDTA, 10% glycerol and 1% protease inhibitor cocktail for plant cell and tissue extracts (Sigma Aldrich, St. Louis, MO) in 50 mM Tris-HCl, pH-8 buffer] to partly thawed tissue (1 ml

g^{-1}). Extract was centrifuged at 14000 x g for 15 min at 4 °C to remove insoluble debris. Supernatant was aliquoted, stored at −70°C and used latter for immunoblot analysis.

3.4.2. Immunoblot Analysis

Expression of HSP17.6, HSP21 and HSP101 was analyzed at the protein level in the leaves of the *in vitro*-grown 'Arnova', 'Marabel', 'Liseta' and 'Cleopatra' plants. Protein concentrations in extracts were determined according to Bradford (1976). Equal amounts of proteins (10 μg) were mixed with SDS-buffer (Laemmli, 1970), loaded and separated on 15% (HSP18 and HSP21) or 12% (HSP101) polyacrylamide gels (SDS-PAGE). Following electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Blots were processed by modified method of Momčilović and Ristić (2007): (a) incubated overnight at 4 °C in blocking solution [10% non-fat dry milk (NFDM) in phosphate-buffered saline with addition of 0,05% Tween 20 (T-PBS)], (b) rinsed two times in T-PBS, 1 min. each wash, (c) incubated 2h at room temperature (RT) in the solution of primary antibody, (d) washed five times in T-PBS (2 x 1 min, 3 x 10 min.), (e) incubated 2h at room temperature (RT) in the solution of secondary antibody, and (f) washed five times in T-PBS (2 x1 min, 3 x 10 min.). The blots were probed with rabbit polyclonal anti-HSP17.6 (raised against *Arabidopsis thaliana* HSP17.6 CI recombinant protein), rabbit polyclonal anti-HSP21 (raised against *Arabidopsis thaliana* chl HSP21 recombinant protein), rabbit polyclonal anti-HSP101 (raised against recombinant HSP101 N-terminal derived from the sequence of *Arabidopsis thaliana* HSP101 protein) or rabbit anti-Cu/ZnSOD (raised against *Arabidopsis thaliana* Cu/ZnSOD recombinant protein) primary antibodies (Agrisera AB, Sweden). Secondary antibodies used in this work were diluted (1: 12500) goat anti-rabbit IgG-HRP (A0545; Sigma Aldrich, St. Louis, MO, USA). Protein bands were detected by Enhanced Chemiluminescence Method (ECL; Vettermann et al., 2002). The expression levels of HSP17.6, HSP21, HSP101 and Cu/ZnSOD were estimated by determining the band volume with ImageQuant software (ver. 5.2, Molecular Dynamics, Sunnyvale, CA).

3.5. Statistical Analysis

Statistical analysis was performed using STATISTICA 8 (StatSoft, Inc. 1984-2007, USA). In order to determine statistically significant differences between the means the data were subjected to one-factor analysis of variance (ANOVA), and the means were separated by Fisher's-LSD multiple range test at a confidence levels: $p<0.05$, $p<0.01$ and $p<0.001$.

4. RESULTS

4. RESULTS

4.1. Relative Heat-Tolerance of *Ex Vitro*-Grown Potato Cultivars

Electrolyte leakage assay was used to evaluate relative heat tolerance of six commercial potato cultivars: ‘Agria’, ‘Arnova’, ‘Liseta’, ‘Laura’, ‘Marabel’ and ‘Cleopatra’ grown *ex-vitro*. This assay measures the release of electrolytes into the surrounding solution which correspondents to HS-induced damage of cellular membranes (CMD). CMD correlates with cultivar/genotype relative heat tolerance (Savić *et al.*, 2012).

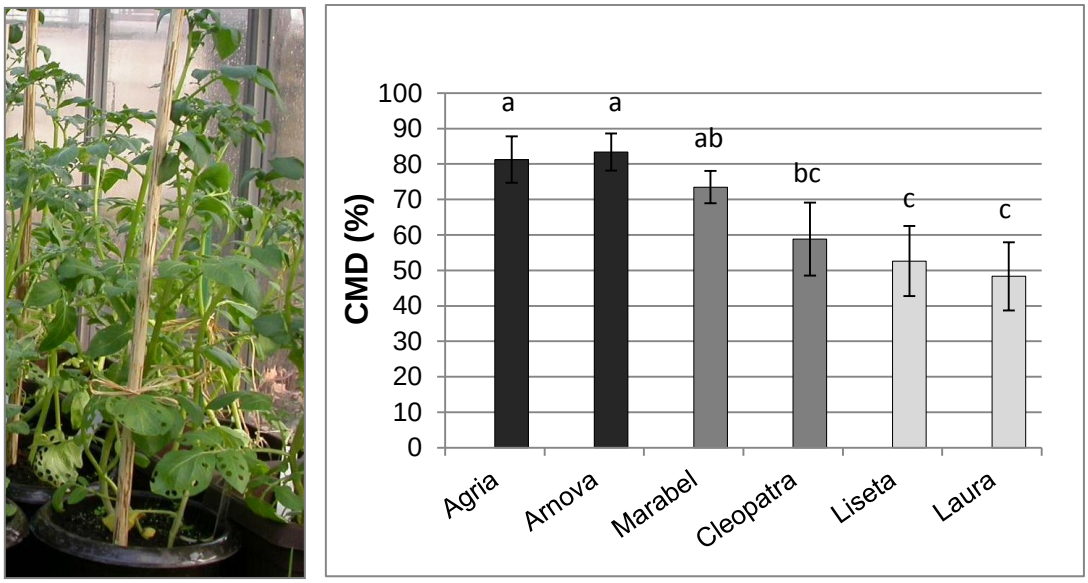

Fig. 9 Cellular membrane damage (CMD) of six potato cultivars determined by the electrolyte leakage assay of heat treated leaf discs. All data are means of four experiments (each with 20 leaf discs per treatment). Means labeled by different letters are significantly different at confidence level $p<0.05$ as determined by Fisher’s LSD test.

Significant difference in cellular membrane damage under HS has been observed between most of the genotypes (Fig. 6). According to results of ELA, analyzed cultivars could be divided into three groups. ‘Laura’ and ‘Liseta’ have shown low values for CMD of 48.3% and 52.6%, respectively, suggesting a relatively high level of membrane termostability and relative heat tolerance. ‘Arnova’ and ‘Agria’ exhibited significantly higher CMD values of 83.4% and 81.2%, respectively, compared to ‘Laura’ and ‘Liseta’. These cultivars/genotypes were designated as relatively heat sensitive. Two other cultivars, ‘Cleopatra’ and ‘Marabel’, exhibited similar CMD values. Also,

'Cleopatra' CMD value was not significantly different from the values in heat tolerant group, while 'Marabel' CMD value was similar to the values of the heat sensitive group. Based on these results, it was not possible to determine with certainty heat tolerance of 'Cleopatra' and 'Marabel' cultivars.

4.2. Potato *In Vitro* Culture

Based on difference in heat tolerance determined by ELA, heat sensitive 'Arnova', heat tolerant 'Liseta', mediate sensitive 'Marabel' and mediate tolerant cultivar 'Cleopatra' were selected for *in vitro* studies. *In vitro* shoot cultures of 4 potato cultivars were successfully established (Fig. 10 a, b). Protocol for surface sterilization of potato sprouts proved to be good procedure for obtaining explants with low level of bacteria/fungi contamination (<5%) and high survival rate (>90%). Shoots were developing from apical or axillary buds of sprout segments on BM with 0.5 mg l^{-1} BA (Fig. 10 a, b). Microplants obtained from these shoots were subcultured using single-node stem cuttings (SNC) as explants (Fig. 10 c).

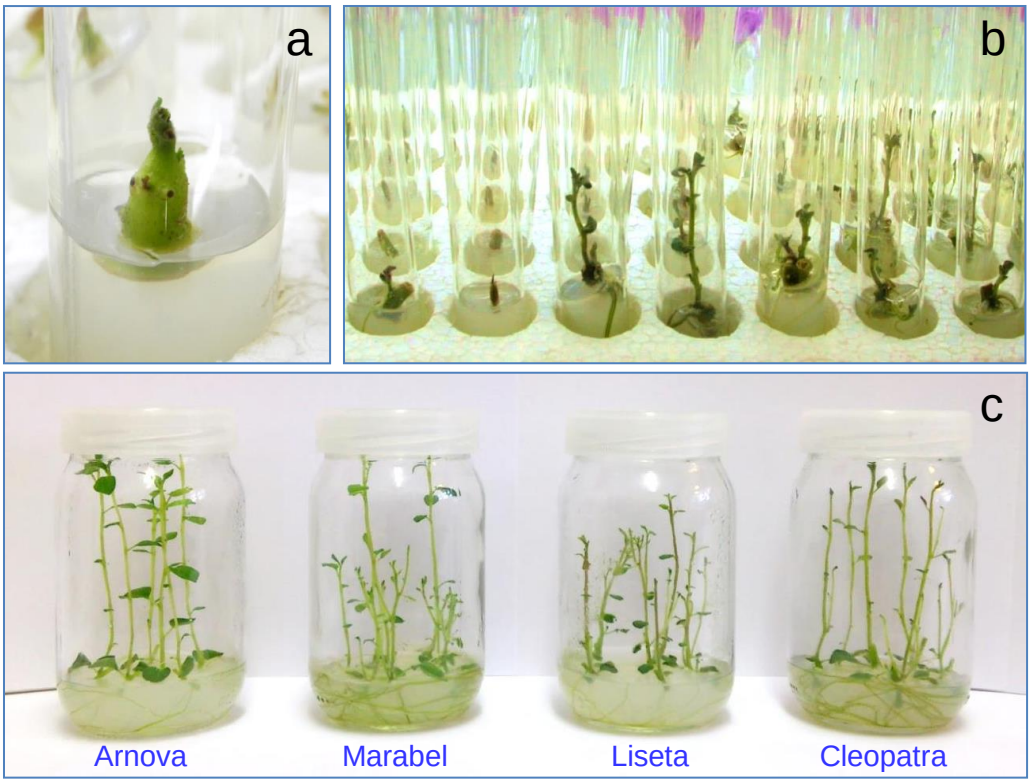

Fig. 10 Potato *in vitro* culture. Development of apical bud from surface sterilized sprout cutting (a). Shoots developed from apical or axillary buds of sprout cuttings after 30 days on BM supplemented with 0.5 mg l^{-1} BA (b). *In vitro* propagation on BM (c).

4.3. Effects of SA on Morphology of Potato Plants at Standard Growth Temperature (23°C)

Effects of SA on potato shoot development were first examined at temperature regularly used for potato *in vitro* propagation (23 °C).

SNC survival and plantlets development from SNC were not affected by SA pretreatment or SA treatment at this temperature; all explants survived and gave rise to plants. However, shoot growth in most of examined cultivars was affected by SA pretreatments (Fig. 11).

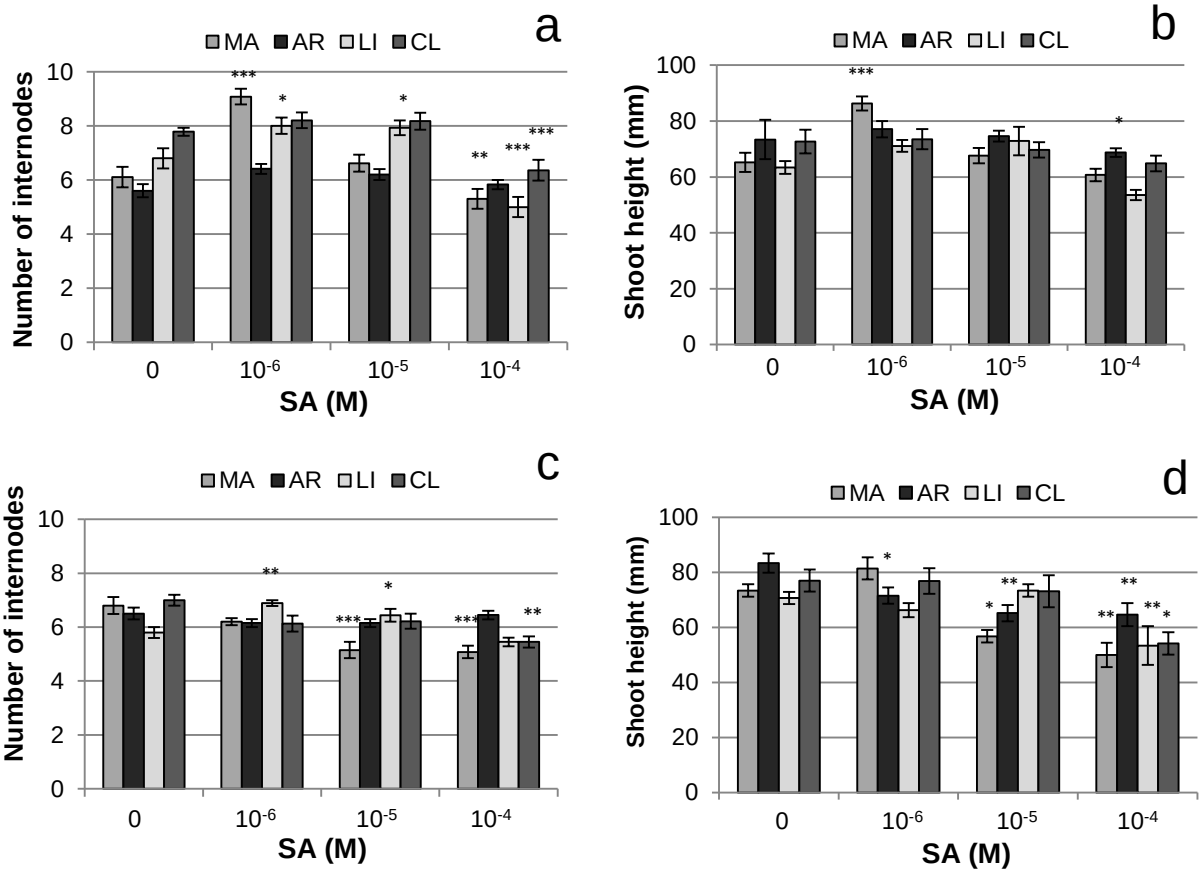

Fig. 11 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato shoot growth at 23 °C. All experiments were repeated twice with 20 SNC per treatment. Asterisks indicate significant difference from control (SA 0 M) for each cultivar separately (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

SA pretreatments did not affect the number of internodes in cv. 'Arnova' (Fig. 11 a). The highest SA concentration (10^{-4} M) caused decline in the internodes number in all examined cultivars, except 'Arnova'. In 'Marabel', 10^{-6} M SA pretreatment had stimulatory effect on the number of internodes. In 'Liseta' both 10^{-6} M and 10^{-5} M SA pretreatments caused increase in the number of internodes, while cv. 'Cleopatra' did not respond on these SA pretreatments.

SA pretreatments mainly did not affect shoot height in examined cultivars (Fig. 11 b), except in two cases: in 'Marabel', 10^{-6} M SA pretreatment had stimulatory effect, while in 'Cleopatra', 10^{-4} M SA pretreatment had inhibitory effect on shoot elongation.

SA treatments had similar effects on the number of internodes in 'Arnova', 'Cleopatra' and 'Liseta' to those observed in SA pretreatments with particular SA concentration: 'Arnova' did not respond on SA, decline in the number of internodes was observed in 'Cleopatra' at 10^{-4} M SA, while increase was observed in 'Liseta' at both 10^{-6} M and 10^{-5} M SA (Fig. 11 c). In 'Marabel', 10^{-5} M and 10^{-4} M SA treatment had inhibitory effect on the number of internodes. Treatment with the highest SA concentration (10^{-4} M) caused decline in shoot height in all examined cultivars (Fig. 11 d). Also, decline in shoot height was observed in 'Arnova' at 10^{-6} M and 10^{-5} M SA, as well as 'Marabel' at 10^{-5} M SA.

SA pretreatments did not affect the number of initiated roots in cvs. 'Arnova' and 'Cleopatra' (Fig. 12 a). In 'Marabel', 10^{-5} M and 10^{-4} M SA pretreatment had inhibitory effect on the number of roots. In 'Liseta', 10^{-6} M and 10^{-5} M SA stimulated root initiation. All SA pretreatments caused significant decline in root length in cv. 'Arnova' (Fig. 12 b). Also, decline in root length was observed in 'Marabel' at 10^{-5} M and 10^{-4} M SA, as well as 'Cleopatra' at 10^{-4} M SA. SA pretreatments did not affect root length in cv. 'Liseta'.

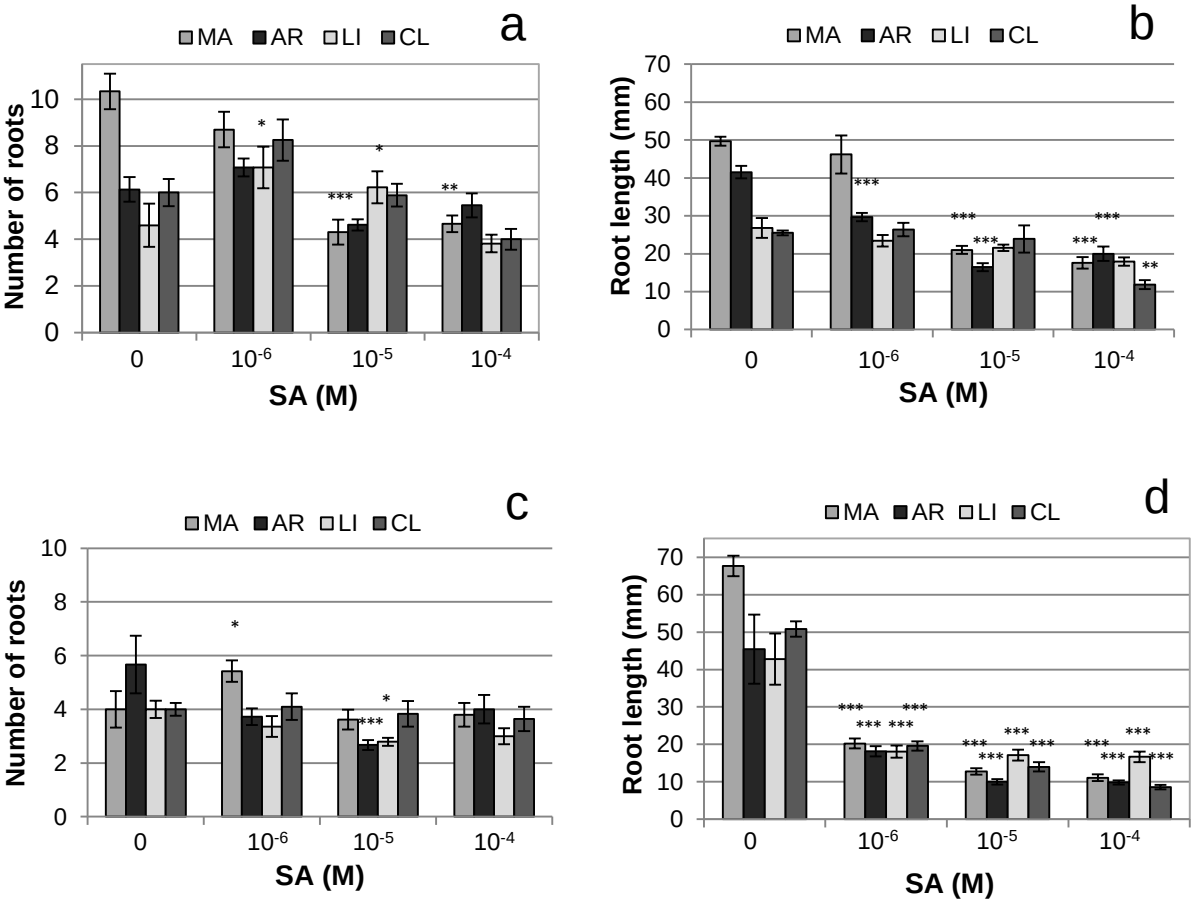

Fig. 12 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato root growth at 23 °C. All experiments were repeated twice with 20 SNC per treatment. Asterisks indicate significant difference from control (SA 0 M) for each cultivar separately (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

SA treatments did not affect the number of initiated roots in cv. 'Cleopatra' (Fig. 12 c). Treatment with 10^{-6} M SA caused increase in root number in 'Marabel', while 10^{-5} M SA treatment had inhibitory effect on root initiation in cvs. 'Arnova' and 'Liseta'. Interestingly, SA treatments had strong inhibitory effect on root elongation (root length) in all examined potato cultivars.

4.4. Effects of SA on Morphology of Potato Plants – Short-Term Heat Stress

SNC survival and plantlets development from SNC were not affected by short-term heat stress at 45 °C, 6h. Control explants, SA-pretreated-explants and SA-treated-explants were showing similar percent of developed shoots after HS-recovery period (Fig. 13).

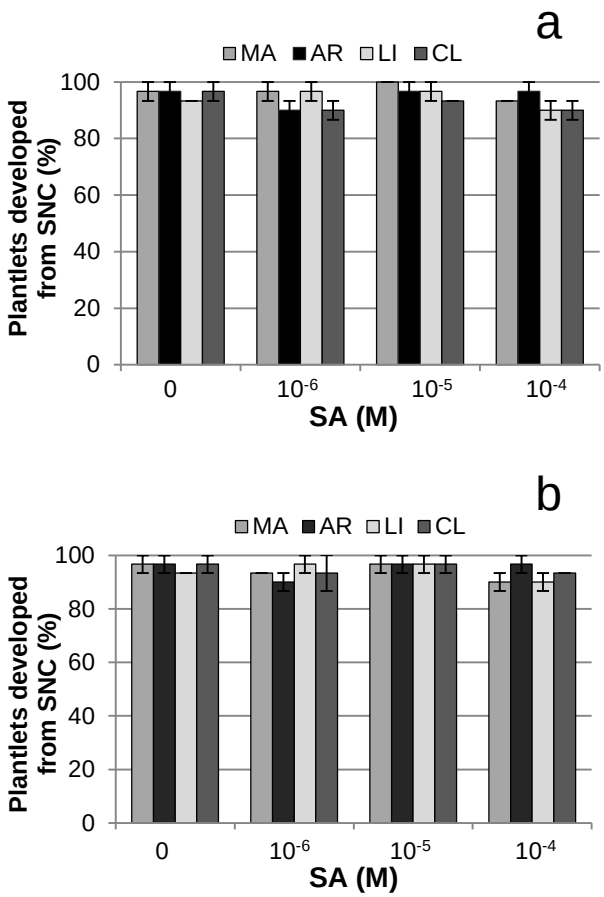

Fig. 13 Effects of SA pretreatment (**a**) and treatment (**b**) on the number of plantlets developed from SNC during recovery period after exposure to 45 °C (6 h). All experiments were repeated twice with 24 SNC per treatment. Asterisks indicate significant differences from controls (SA 0 M) for each cultivar separately (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

SA pretreatments did not affect shoot growth (number of internodes and shoot height) in examined cultivars under HS and HS recovery period, except in cv. 'Liseta' where decline in the number of internodes and shoot height was observed at 10^{-6} M SA (Fig. 14 a, b). However, when SA was continuously applied (SA treatment), increase in the number of internodes was observed in 'Arnova' at 10^{-5} M SA, 'Liseta' at 10^{-6} M and

10^{-5} M SA, as well as 'Cleopatra' at 10^{-5} M and 10^{-4} M SA (Fig. 14 c, d). Also, all examined SA concentrations caused increase in shoot height in 'Arnova' and 'Cleopatra'. Increase at 10^{-6} M SA was observed in 'Liseta'. In 'Marabel', SA treatments caused decline in the number of internodes and 10^{-4} M SA significantly reduced shoot height.

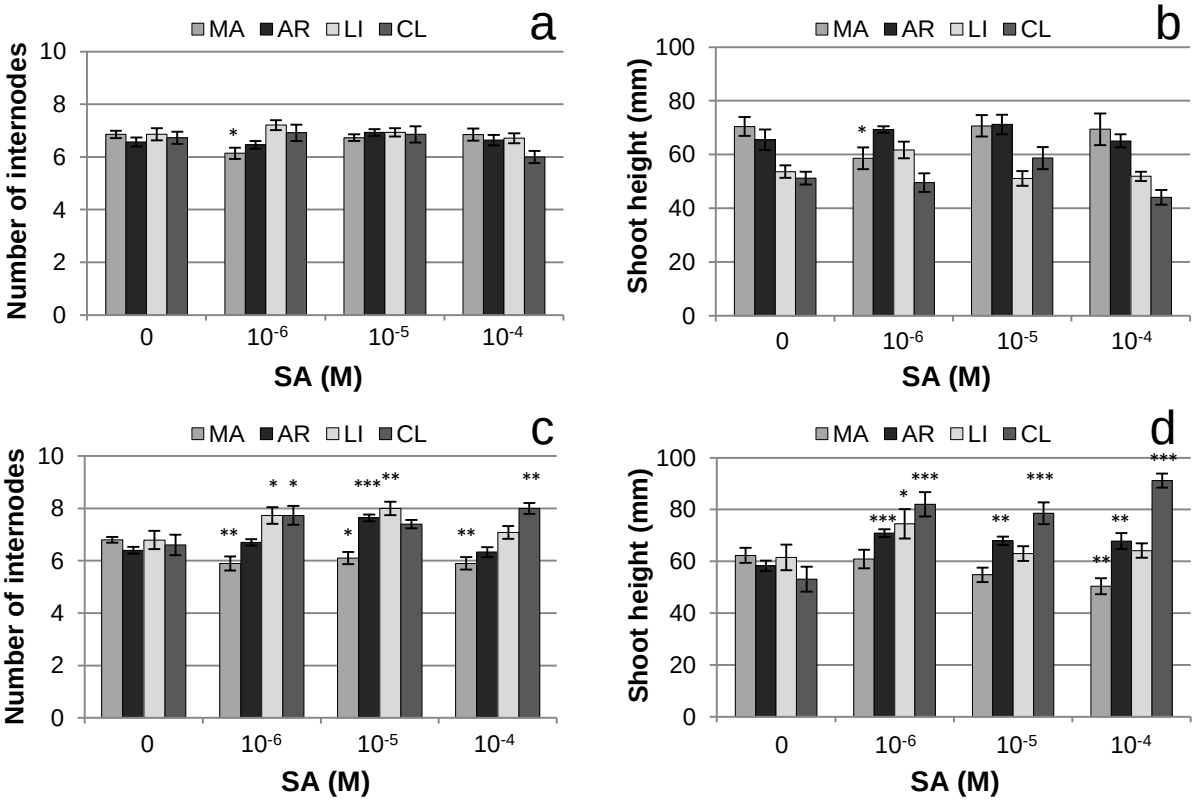

Fig. 14 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato shoot growth during HS and HS-recovery period. All experiments were repeated twice with 20 SNC per treatment. Asterisks indicate significant difference from control (SA 0 M) for each cultivar separately (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

SA pretreatments did not affect root initiation in 'Arnova', while they caused decline in the number of initiated roots in 'Marabel' during HS and HS-recovery period (Fig. 15 a, b). Decline in the number of roots was also observed in 'Cleopatra' at 10^{-6} M and 10^{-4} M SA Conversely, pretreatment with 10^{-5} M SA caused increase in the number of initiated roots in 'Liseta'. SA pretreatments did not affect root elongation (root

length) in cv. ‘Arnova’. However, SA pretreatments caused decrease in the root length in all other cultivars, with the exception of 10^{-4} M SA-pretreatment that had no effect on root elongation in cv. ‘Cleopatra’. SA treatments reduced root number in cvs. ‘Marabel’ and ‘Arnova’ (Fig. 15 e) and caused significant decline in the root length in all examined cultivars (Fig. 15 d).

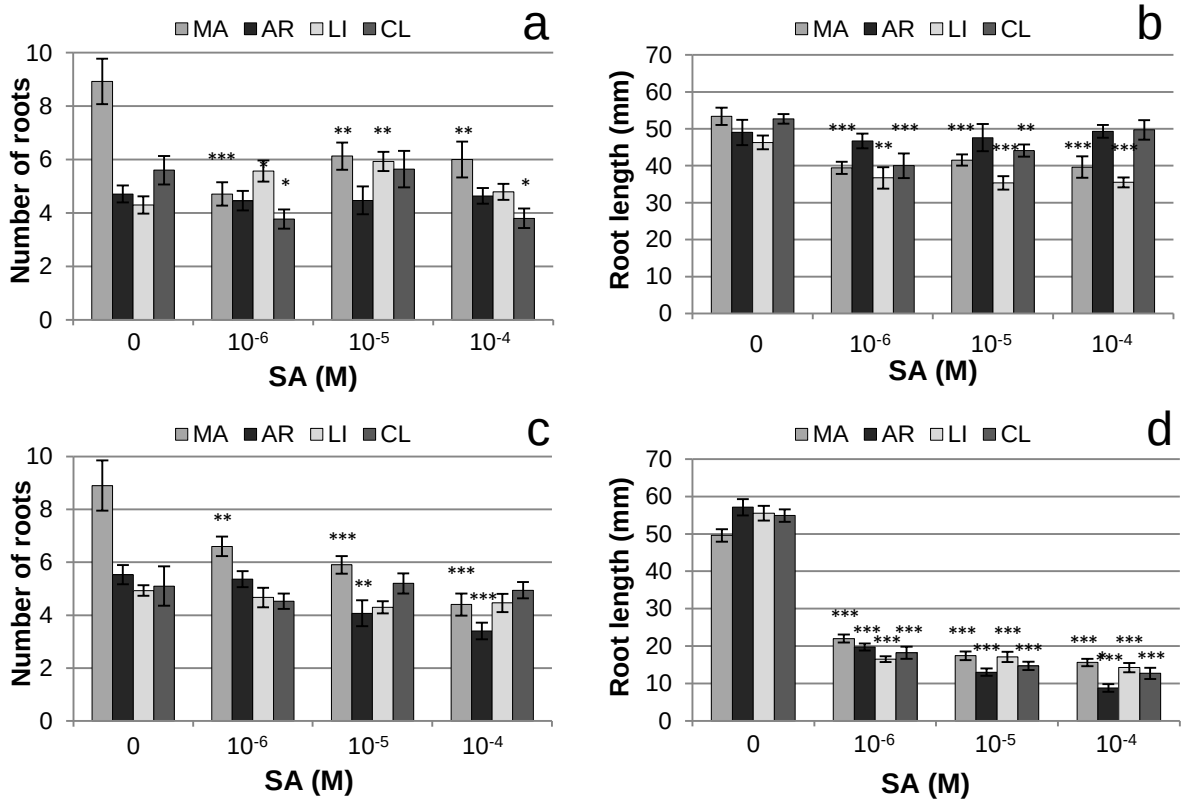

Fig. 15 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato root growth during HS and HS-recovery period. All experiments were repeated twice with 20 SNC per treatment. Asterisks indicate significant difference from control (SA 0 mM) for each cultivar separately (*, p<0,05; **, p<0,01; ***, p<0,001). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

4.5. Effects of SA on Morphology of Potato Plants – Long-Term Heat Stress

Effects of SA pretreatments and SA treatments on potato shoot and root development and growth were also examined after long-term HS at 35 °C (20 days). Although necrosis of the explants was rarely observable, development of significant percent of

axillary buds (44-80%) was arrested in SA-non-treated controls. SA pretreatment with 10^{-5}M SA caused 17.5%, 25.8% and 28.6% increase of plants developed from SNC in cvs. 'Marabel', 'Arnova' and 'Cleopatra', respectively, while 10^{-6} M SA pretreatment enhanced plant development for 26.7% in 'Liseta' (Fig 16 a). Pretreatment with 10^{-4} M SA did not affect percent of developed plants in examined cultivars. Treatments with 10^{-5} M and 10^{-4} M SA promoted plant development from SNC in 'Arnova', 'Liseta' and 'Cleopatra', while 10^{-6} M SA pretreatment enhanced plant development in 'Liseta' and 'Cleopatra' (Fig. 16 b). Interestingly, cv. 'Marabel' did not respond to any of SA treatments (Fig 16 b).

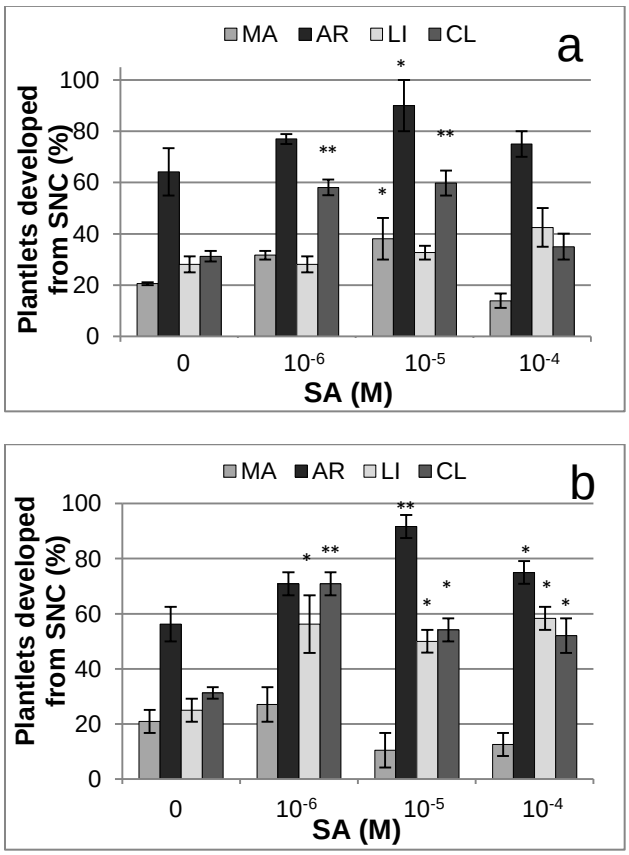

Fig. 16 Effects of SA pretreatment (**a**) and treatment (**b**) on the number of plantlets developed from SNC during long-term exposure to moderately elevated temperature (35 °C, 20 days). All experiments were repeated twice with 24 SNC per treatment. Asterisks indicate significant differences from controls (SA 0 M for each cultivar separately (*, p<0,05; **, p<0,01; ***, p<0,001). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

SA pretreatments did not affect number of internodes and height of developed shoots in examined cultivars exposed to 35 °C (Fig. 17 a, b), except in 'Liseta' where increase in the internodes number was detected at 10^{-6} M and 10^{-5} M SA. Treatment with 10^{-5} M caused increase in the number of internodes in 'Arnova', while 10^{-4} M SA treatment reduced internodes number (Fig. 17 c). The increase in the internode number was observed in 'Liseta' at 10^{-4} M SA, while the number of internodes was reduced in 'Cleopatra' at 10^{-6} M SA.

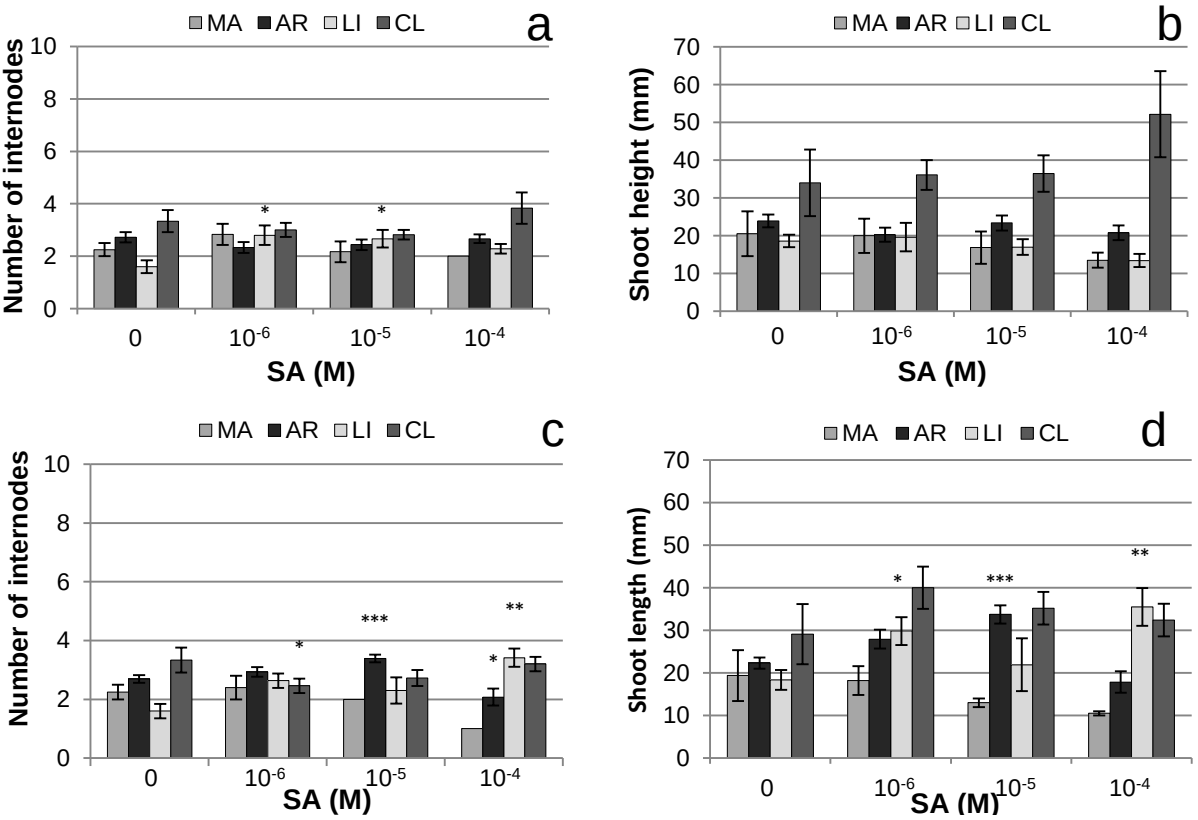

Fig. 17 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato shoot growth during long-term HS. Asterisks indicate significant differences from controls (SA 0 M) for each cultivar separately (*, p<0,05; **, p<0,01; ***, p<0,001). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

Increase in the shoot height was observed in 'Arnova' at 10^{-5} M SA (Figs. 17 d and 18), as well as 'Liseta' at 10^{-6}M and 10^{-4}M SA treatments. In 'Marabel' and 'Cleopatra', SA treatments did not affect number of internodes and shoot height, with the exception of 10^{-4} M SA treatment which significantly reduced shoot height in 'Marabel'.

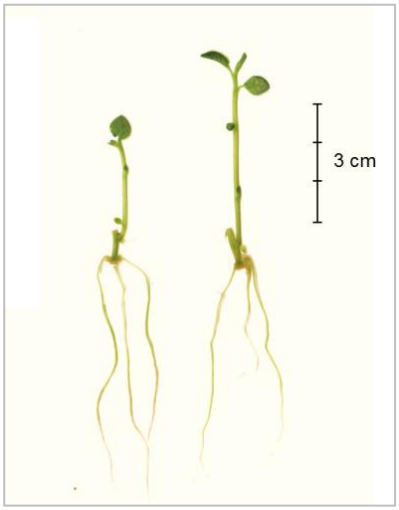

Fig. 18 Control plant (left) and 10^{-5} M SA-treated plant (right) of cv. 'Arnova' after 20 days at 35 °C.

SA pretreatments were not affecting potato root initiation and elongation during 35 °C-HS, except in 'Cleopatra' where increase in root length at 10^{-6} M SA was detected (Fig 19 a, b). SA treatments did not affect root initiation in examined cultivars, with the exception of 10^{-4} M SA-treatment which caused increase in the number of roots in 'Liseta' formed during HS (Fig 19 c). However, all SA treatments caused decline in root elongation in 'Liseta' (Fig 19 d). Decline in root length was observed in 'Marabel' and 'Arnova' at 10^{-4} M SA treatment and 'Cleopatra' at 10^{-5} M and 10^{-4} M SA treatments (Fig. 19 d).

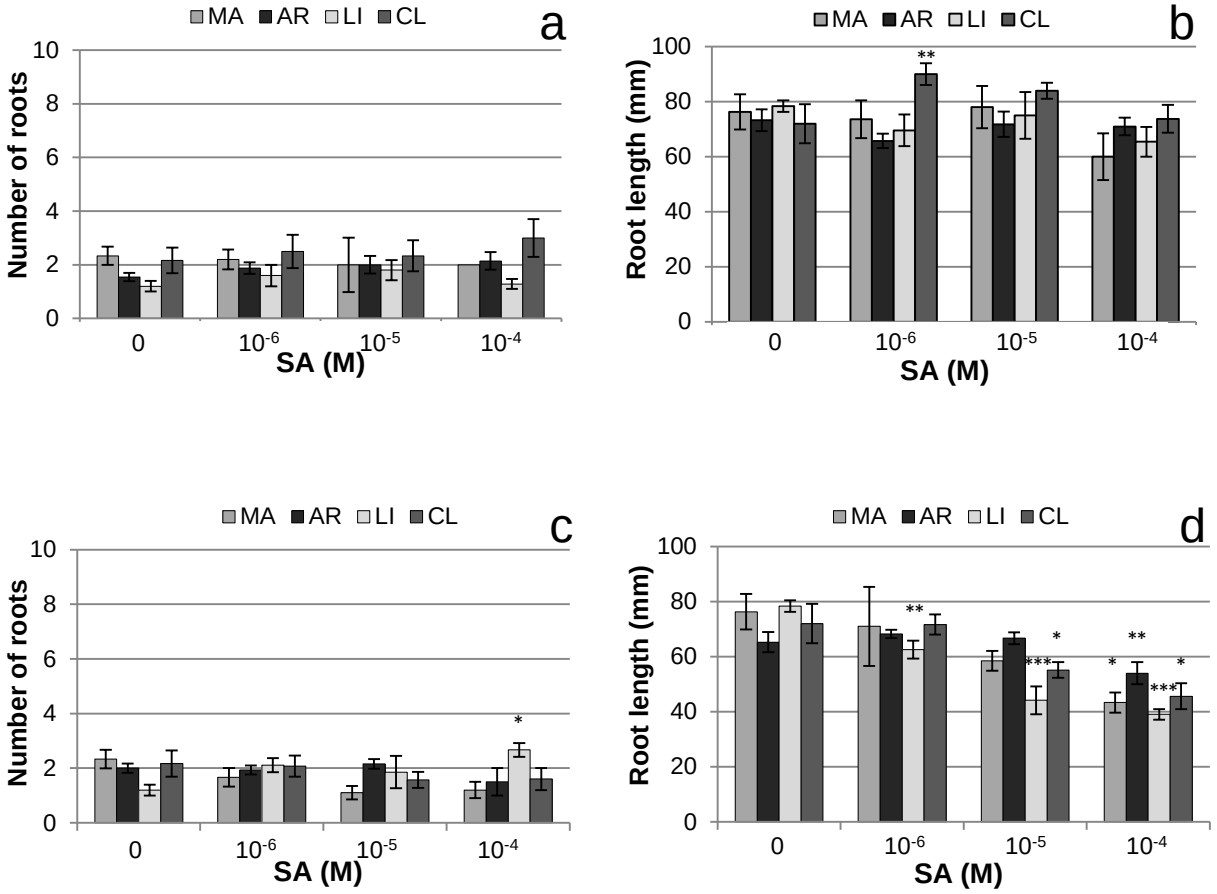

Fig. 19 Effects of SA pretreatment (**a**, **b**) and treatment (**c**, **d**) on potato root initiation and growth during long-term HS. Explants were subjected to 35 °C for 20 days. Asterisks indicate significant differences from controls (SA 0 M) for each cultivar separately (*, p<0,05; **, p<0,01; ***, p<0,001). Potato cultivars: MA - Marabel, AR - Arnova, LI - Liseta, CL - Cleopatra.

4.6. Effects of SA Pretreatment on HSP Expression

To determine whether SA affects HSP accumulation in examined potato cultivars, expression of cytosolic HSP17.6 CI, chloroplast HSP21 and cytosolic HSP101 were investigated in leaves of 10^{-5} M SA-pretreated plantlets exposed for 6 h to 23 °C, 35 °C or 45 °C.

4.6.1. HSP17.6 Expression

Immunoblot analysis has revealed that SA enhances accumulation of potato HSP17.6 under both heat treatments, 35 °C and 45 °C, and also induces minor accumulation of this protein in cv. 'Cleopatra' plants maintained at 23°C (Fig. 20).

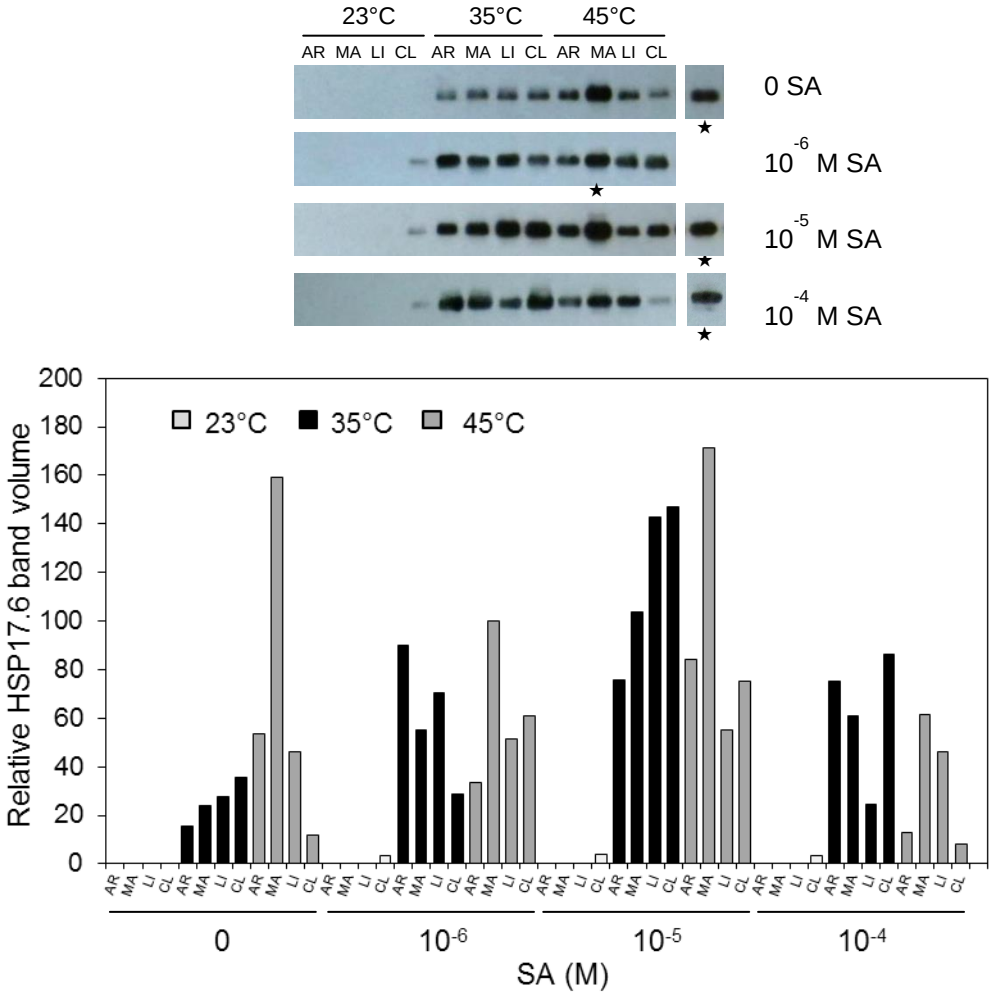

Fig. 20 Effect of SA on the expression and accumulation of HSP17.6 in the potato leaves under heat stress *in vitro*. Potato plants were grown on BM supplemented with 10^{-6} to 10^{-4} M SA. Amounts of accumulated HSP17.6 were presented relatively to the level of HSP17.6 in heat-stressed MA leaves (45 °C, 10^{-6} M SA; labeled by asterisk). Similar results were obtained in a duplicate blot. Potato cultivars: AR - Arnova, MA- Marabel, LI - Liseta, CL - Cleopatra.

The most prominent enhancement of protein accumulation at 35 °C has been observed in 10^{-5} M SA-pretreated plantlets, although some of the cultivars responded also on 10^{-6} M and 10^{-4} M SA pretreatment. As compared to the control plants (SA 0), 10^{-5} M SA-pretreated 'Arnova', 'Marabel', 'Liseta' and 'Cleopatra' plants were, respectively, accumulating 4.8-, 4.3-, 5.2- and 4.1-fold higher amounts of HSP17.6 when exposed to 35°C. The effect of SA was not that prominent at 45°C. The 10^{-5} M SA-pretreated 'Arnova', 'Marabel', 'Liseta' were accumulating 1,6-, 1,1- and 1,2-fold higher amounts of HSP17.6 compared to control plants. The significant, 6.4-fold, increase was observed only in 10^{-5} M SA-pretreated cv. 'Cleopatra', whose control plants were accumulating small amounts of investigated protein at 45 °C. Interestingly, 10^{-4} M SA pretreatment caused decline in HSP17.6 abundance at 45 °C in three examined cultivars: 'Arnova', 'Marabel' and 'Cleopatra'.

4.6.2. HSP21 Expression

The most prominent SA effect on chloroplast HSP21 accumulation has been observed at 45 °C (Fig. 21). Significant decline in HSP21 abundance has been observed at all examined SA concentrations (Fig. 21). For instance, 10^{-6} M-SA-pretreated 'Arnova', 'Marabel', 'Liseta' and 'Cleopatra' plants were, respectively, accumulating 4.19-, 10.02-, 1.51- and 3.62-fold lower amounts of HSP21 compared to non-treated plants when exposed to 45 °C. The inhibitory effect of SA was also observable at 35 °C, although potato plantlets generally accumulated lower amounts of HSP21 during this temperature treatment. The 10^{-6} M SA-pretreated 'Arnova' and 'Liseta' were accumulating 2.93- and 4.77-fold lower amounts of HSP21 at 35 °C compared to control plants (SA 0), while accumulation was not detected in 'Marabel' and 'Cleopatra'. Expression of HSP21 was not detected at 23 °C.

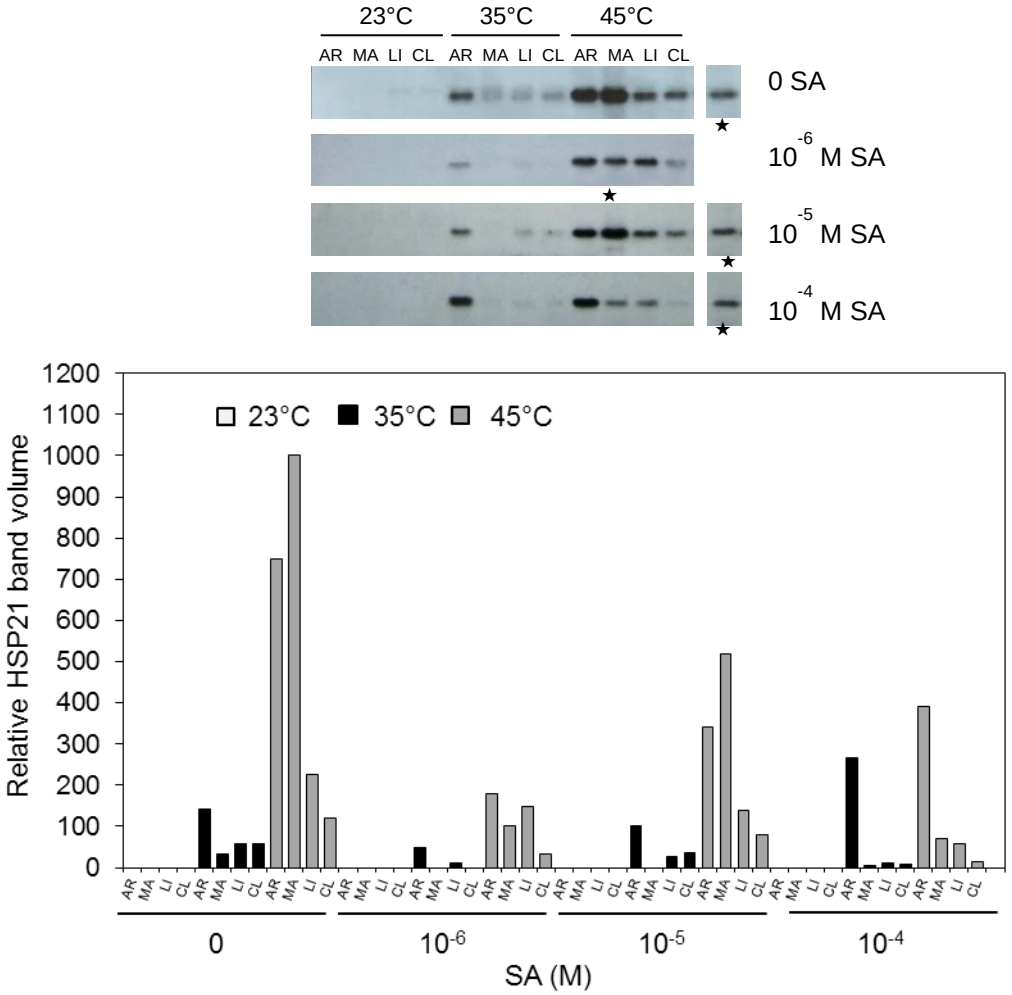

Fig. 21 Effect of SA on the expression and accumulation of HSP21 in the potato leaves under heat stress *in vitro*. Amounts of accumulated HSP21 were presented relatively to the level of HSP21 in heat-stressed MA leaves (45 °C, 10^{-6} M SA; labeled by asterisk). Similar results were obtained in a duplicate blot. Potato cultivars: AR - Arnova, MA- Marabel, LI - Liseta, CL - Cleopatra.

4.6.3. HSP101 Expression

The effect of SA pretreatment on accumulation of constitutively expressed cytosolic HSP101 was also examined at 23 °C, 35 °C or 45 °C. Compared to 23 °C, high temperature treatments (35 °C or 45 °C) generally caused increase in the level of HSP101 (Fig. 22). At 45 °C, increase in HSP101 abundance has been observed in 10^{-6} M SA-pretreated 'Arnova', 'Marabel', 'Liseta' and 'Cleopatra' microplants which were,

respectively, accumulating 1.72, 1.47-, 1.35- and 1.30-fold higher amounts of HSP101 than non-treated plants. Pretreatment with 10^{-6} M SA, did not affected accumulation of this protein at 35 °C. Conversely, 10^{-5} M SA pretreatment did not cause substantial change in HSP101 accumulation at 45 °C, while increase in HSP101 abundance has been observed in 'Marabel' and 'Cleopatra' at 35 °C. Pretreatment with 10^{-4} M SA, on the other hand, caused decline in protein abundance at both 35 °C and 45 °C. At 23 °C, pretreatments with 10^{-6} M and 10^{-5} M SA caused decline in HSP101 abundance, while 10^{-4} M SA completely suppressed HSP101 accumulation in potato plants.

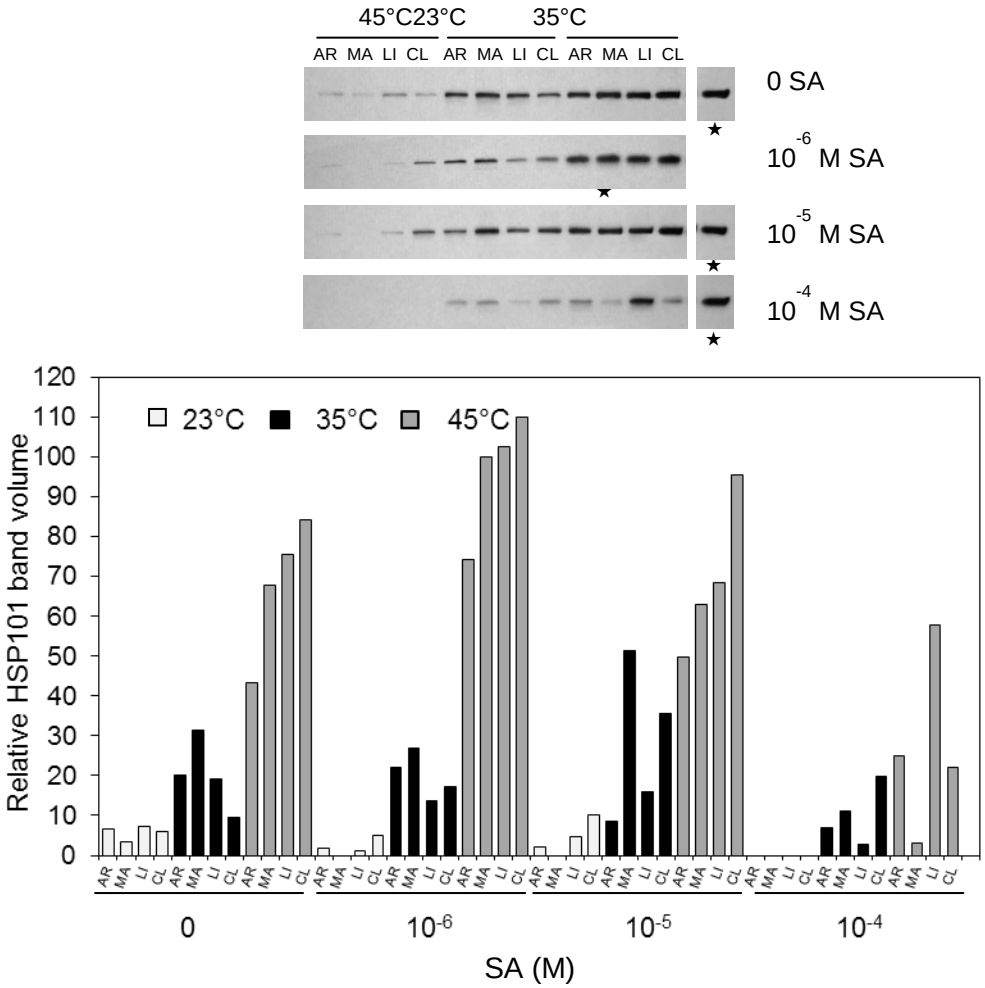

Fig. 22 The effect of SA on the expression and accumulation of HSP101 in the potato leaves under heat stress *in vitro*. Amounts of accumulated HSP101 were presented relatively to the level of HSP101 in heat-stressed MA leaves (45 °C, 10^{-6} M SA; labeled by asterisk). Similar results were obtained in a duplicate blot. Potato cultivars: AR - Arnova, MA- Marabel, LI - Liseta, CL - Cleopatra.

4.7. SA Effect on Cu/ZnSOD Expression

Investigation of chloroplast Cu/ZnSOD revealed that 10^{-5} M SA-pretreatment substantially affects accumulation of this protein in potato at all tree temperature treatments (Fig. 23). At 23 °C, 10^{-5} M-SA pretreated plants of cvs. 'Arnova', 'Marabel', 'Liseta' and 'Cleopatra' were accumulating, respectively, 1.32-, 2.61-, 2.25- and 3.27-fold higher amounts of Cu/ZnSOD than non-treated plants.

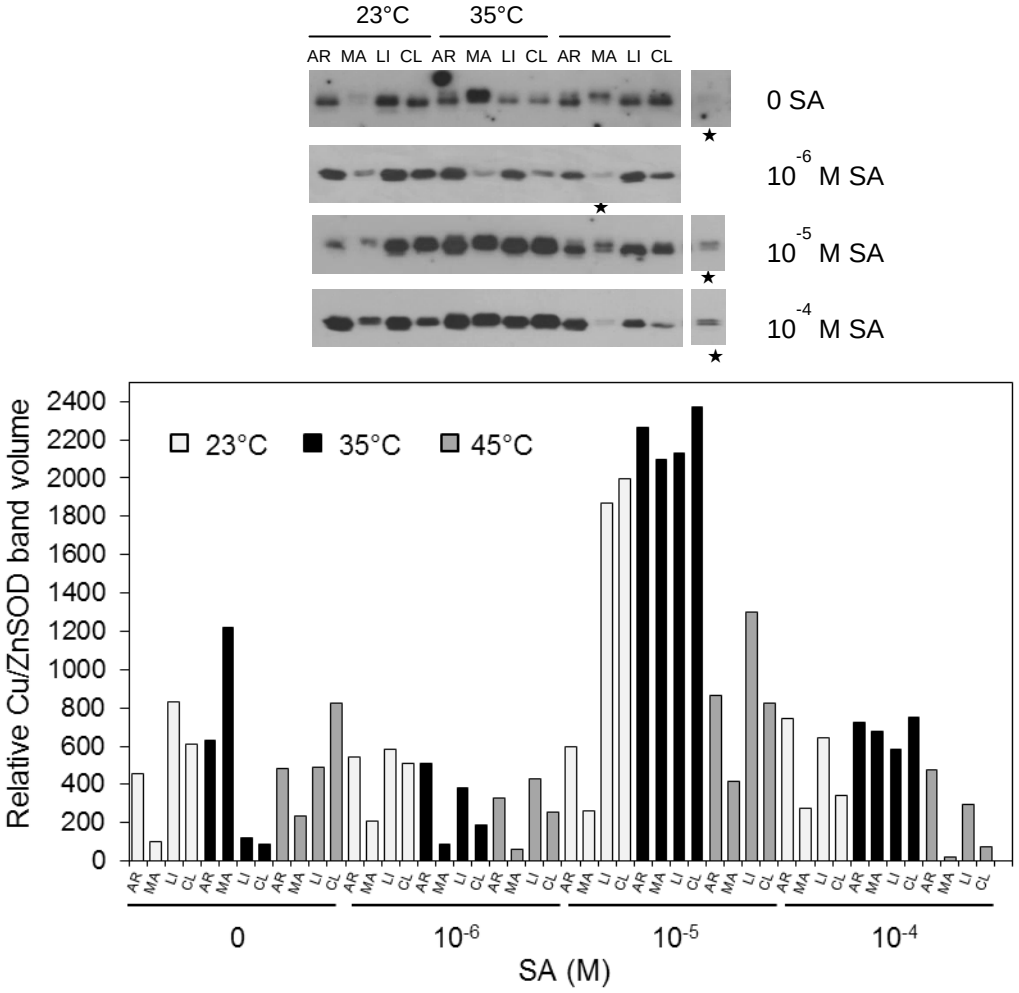

Fig. 23 The effect of SA on the expression and accumulation of Cu/ZnSOD in the potato leaves under heat stress *in vitro*. Amounts of accumulated Cu/ZnSOD were presented relatively to the level of HSP Cu/ZnSOD in heat-stressed MA leaves (45 °C, 10^{-6} M SA; labeled by asterisk). Similar results were obtained in a duplicate blot. Potato cultivars: AR - Arnova, MA- Marabel, LI - Liseta, CL - Cleopatra.

This effect was even more prominent at 35 °C where four cultivars also exhibited considerably higher level of Cu/ZnSOD; 'Arnova', 'Marabel', 'Liseta' and

'Cleopatra' microplants accumulated, respectively, 3.61-, 1,72-, 17.6- and 26.39-fold higher amounts of Cu/ZnSOD than non-treated plants. At 45 °C, 10^{-5} M-SA pretreated 'Arnova', 'Marabel' and 'Liseta' microplants were accumulating 1.80-, 1.75- and 2.65-fold higher amounts of Cu/ZnSOD, respectively, while no increase was detected in 'Cleopatra'.

4.8. HSP17.6 Expression at Different Time Points after Cessation of SA Treatment

To investigate duration of the SA effect on potato HSP17.6 accumulation during HS (45 °C, 6 h), expression of this protein was investigated at different time points after cessation of 10^{-5} M SA pretreatment (0, 1, 3, 7, 10 or 14 days).

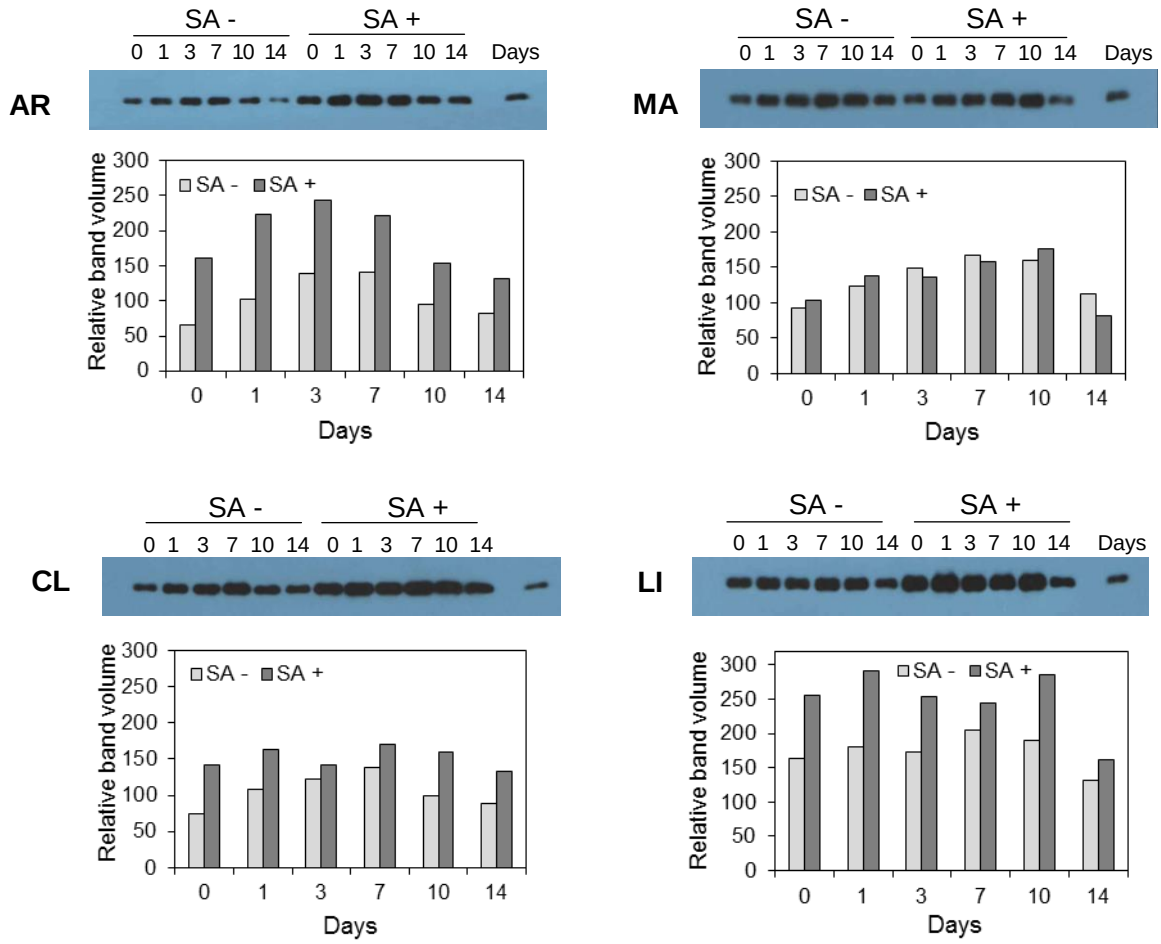

Fig. 24 HSP17.6 expression in leaves of potato under HS (45 °C, 6 h) at different time points after cessation of 10^{-5} M SA pretreatment. Potato cultivars: AR - Arnova, MA - Marabel, LI - Liseta, CL - Cleopatra. SA+ - SA-pretreated plants, SA − - control plants.

In leaves of three investigated cultivars, ‘Arnova’, ‘Liseta’ and ‘Cleopatra’, stimulating effect of SA on accumulation of HSP17.6 under HS was detectable during period of 14 days after cessation of SA pretreatment, while in ‘Marabel’, similar amounts of HSP17.6 were produced and accumulated in leaves of SA-pretreated (SA+) and non-treated (SA-) plantlets (Fig. 24). In cvs. ‘Arnova’, ‘Liseta’ and ‘Cleopatra’, the level of accumulated HSP17.6 gradually increased in leaves of both SA+ and SA- microplants up to 7-10 days after cessation of SA application, and declined afterwards. Stimulating effect of SA on HSP17.6 accumulation in ‘Arnova’, ‘Liseta’ and ‘Cleopatra’ was the weakest 14 days after application.

5. DISSCUSION

5. DISCUSSION

5.1. Relative Heat-Tolerance of Potato Cultivars Grown *Ex Vitro*

Cell membranes represent one of the first targets of heat stress, and maintenance of cell membrane integrity during high-temperature exposure represents important component of heat tolerance in plants (Wahid et al., 2007b). The degree of cell membrane damage induced by HS can be easily assessed through measurements of electrolyte leakage from the cells by ELA. ELA is relatively simple, reproducible and fast procedure which allows fast analysis of considerable number of samples. So far, this assay has been used as an quantitative measure of heat tolerance in diverse plant species, including soybean (Martineau *et al.*, 1979), tomato (Chen *et al.*, 1982), cotton (Ashraf *et al.*, 1994), sorghum (Marcum, 1998), wheat (Blum *et al.*, 2001), barley (Wahid and Shabbir, 2005) and potato (Ahn *et al.*, 2004; Savić *et al.*, 2012).

In this study, ELA was used to estimate relative heat-tolerance of six, *ex vitro*-grown potato cultivars: 'Agria', 'Arnova', 'Liseta', 'Laura', 'Marabel' and 'Cleopatra'. The procedure included exposure of potato leaf discs to high temperature (50 °C) and subsequent determination of the degree of cell membrane injury (CMD) through measurement of electrolyte leakage. Significant differences in cell membrane integrity have been observed between analyzed potato cultivars/genotypes. Cvs. 'Laura' and 'Liseta' were showing a relatively high level of membrane termostability and relative heat tolerance, while 'Arnova' and 'Agria' were designated as relatively heat sensitive. The heat tolerances of two other cultivars, 'Cleopatra' and 'Marabel', were not possible to determine with certainty**.** These results were in concordance with previous findings of Savić *et al.* (2012), with the exception of cv. 'Liseta' that exhibited higher thermotolerance than previously detected. In order to examine in more details potato heat tolerance, *in vitro* cultures of cultivars 'Arnova', 'Liseta', 'Marabel' and 'Cleopatra' were established, and used in further investigations of SA effects on morphology and HSP expression under HS.

5.2. Effects of SA on Potato Morphology *In Vitro*

The effects of SA on plant morphology in cultivars 'Arnova', 'Liseta', 'Marabel' and 'Cleopatra' were firstly examined at temperature regularly used for potato *in vitro* propagation (23 °C). Following parameters were measured: percent of developed plantlets from SNC, shoot length, number of internodes, number of roots and root length. Number of internodes and shoot length are parameters that illustrate, respectively, process of shoot internode initiation and internode elongation. Number of roots is parameter that depicts process of root initiation, while root length illustrates process of root elongation.

Effects of SA on shoot growth in potato depend on cultivar/genotype, SA concentration and duration of application. Depending on cultivar, concentrations of 10^{-6} M and 10^{-5} M SA either stimulated internode initiation or did not affect this process, while 10^{-4} M SA mainly inhibited internode initiation. Internode elongation (contributing to the shoot height) was mostly not affected by SA pretreatment. Continuous SA application, however, inhibited internode elongation in all cultivars at the highest concentration used (10^{-4} M), as well as 'Marabel' and 'Arnova' at 10^{-5} M. In plants, most of the PGRs shows stimulatory (or inhibitory) effect on certain physiological process within defined range of concentrations and may show opposite effect if the concentrations are too high. This was already determined for SA effects on seed germination (Rajjou *et al.*, 2006; Xie *et al.*, 2007), nitrate reductase activity (Fariduddin *et al.*, 2003; Hayat *et al.*, 2005) and chlorophyll content (Hayat *et al.*, 2005). Results of this study suggest that lower SA concentrations (10^{-6}M-10^{-5}M) have stimulatory effect on process of internode initiation, while relatively high concentration (10^{-4} M) inhibits this process in most potato cultivars. Also, internode initiation and internode elongation were showing different sensitivity regarding SA. Internode elongation was affected in all examined cultivars only during continuous 10^{-4} M SA application.

SA also affected rooting of potato plants *in vitro*. Effects of both SA pretreatments and SA treatments on root initiation were not consistent. Depending on cultivar and concentration, SA stimulated or inhibited the number of initiated roots. On the other hand, both SA pretreatments and SA treatments caused decline in root length.

Inhibitory effect was especially noticeable in SA treatments; when continuously applied, all investigated SA concentrations (10^{-6} M -10^{-4} M) inhibited root elongation in 'Arnova', 'Liseta', 'Marabel' and 'Cleopatra'. So far, stimulatory effects of SA on the root growth have been observed in soybean (Gutiérrez-Coronado *et al.*, 1998), maize (Khodary, 2004), *Chrysantemum morifolium* (Villanueva–Couoh *et al.*, 2009) and *Pinus patula* (San-Miguel *et al.*, 2003). In soybean, SA stimulated root elongation in the broad range of applied concentrations (10^{-8} - 10^{-2} M). Significant increase in root length (28–30%), root fresh weight (33%) and root dry weight (45 to 54%) were detected after applications of relatively low SA concentrations (10^{-8} - 10^{-6} M) in *Pinus patula* plants (San-Miguel *et al.*, 2003). However, these results were obtained under considerably different experimental conditions compared to potato *in vitro* culture. SA was mainly applied by leaf spraying on plants grown in greenhouse or field. Findings of Echevarria-Machado *et al.* (2007) on *Catharanthus roseus* transformed root cultures (hairy roots) suggest that even SA concentrations as low as 10^{-16} M increases the number of emerged roots and root fresh weight when added to the culture medium. There is a possibility that SA concentrations used in this study were mainly too high to stimulate root elongation, whereas they inhibited this process in examined cultivars/genotypes.

5.3. Effects of SA on Potato Morphology under Heat Stress

Most commercial potato cultivars were developed for growing in temperate climate regions and they are highly sensitive to elevated temperatures. Air temperature of 30 °C can cause heat stress in some potato genotypes (Levy and Veilleux, 2007), while in maize temperatures of 40 °C and above triggers HS (Momčilović and Ristić, 2007). In this study, SNC were exposed to 35 °C long-term temperature treatment without acclimation in order to investigate possible effects of SA on the basal heat tolerance of potato cultivars. Besides, it was intriguing to investigate whether SA affects (promotes) basal thermotolerance of potato under severely elevated temperature – 45 °C. Experiments were conducted *in vitro* since this experimental setup allowed investigation under strictly controlled conditions, with variation of only selected factors – temperature and SA concentration.

Interestingly, short exposure (6 h) to excessively elevated temperature was not severe enough to cause necrosis of the explants or significantly arrest development of axillary buds, which gave rise to shoots in all four examined cultivars during 20-day long recovery period. On the other hand, prolonged exposure (20 days) to moderately elevated temperature (35 °C) substantially reduced the number of plantlets developed from SNC. Although necrosis of the explants was rarely observable, arrested bud development was detected in significant percent (44-80%) in SA-non-treated controls. Extensive necrosis of explants, ranging from 83-87%, was observed after *S. tuberosum* cv. 'Atlantic' exposure to both short-term (42 °C, 15.25 h) and long-term (35 °C, 5-7 weeks) heat treatments (López-Delgado *et al*., 1998), indicating that cultivars used in our study were more thermotolerant.

SA pretreatments mainly stimulated plant development from SNC during long-term HS (35 °C). SA protective effect was more prominent in SA treatments; all applied SA concentrations increased percent of developed plantlets from SNC in three cultivars, while cv. 'Marabel' did not respond on SA treatment. Difference in genotype sensitivity to SA has been observed in the study of López-Delgado *et al.* (2004) where survival rates of seven potato cultivars were investigated after HS *in vitro*. Pretreatment with 10^{-5} M SA, before exposure to 42 °C for 30 days, resulted in the 40–73% increase in the microplant´s survival rate in five cultivars, 10% decline in one the cultivars, while one cultivar was not SA-responsive (López-Delgado *et al.*, 2004).

The processes of internode initiation and internode elongation were more affected by long-term exposure to moderately elevated temperature of 35 °C than short exposure to 45 °C; substantial reduction in the number of internodes and shoot length was detected at 35 °C compared to 23 °C. SA pretreatments mainly did not affect internode initiation and elongation at 35 °C and during 45 °C-recovery period. SA treatments, however, induced significant increase in internodes number and shoot height of all cultivars except 'Marabel' where decline in values of both parameters were observed during 45 °C-recovery period. Inhibitory effect of SA treatment (10^{-4} M) on 'Marabel' internode initiation and elongation was also detected at 35 °C, while these treatments had either stimulating effect or did not affect shoot growth in other cultivars.

These results suggest that effects of SA on potato shoot growth under HS depend on cultivar/genotype and duration of application.

SA affected processes of root initiation and root elongation in plantlets developed from SNC after exposure to 45 °C. SA pretreatments caused decline in the number of roots and root length of plantlets in examined cultivars, with the exception of 'Marabel'. SA treatments caused decline in the number of roots in two cultivars, and had prominent inhibitory effect on root elongation in all examined cultivars. Results considering SA inhibitory effect on root elongation were similar to ones obtained at 23 °C. This might be explained by the long recovery period of 20 days at 23 °C which possibly prevailed effects of short exposure to high temperature (45 °C, 6 h).

Interestingly, SA pretreatments did not affect processes of root initiation and root elongation of potato plants during long-term exposure to 35° C, with the exception of cv. 'Cleopatra' where stimulatory effect of 10^{-5} M SA was observed. Also, SA treatments did not affect processes of root initiation at 35 °C, except in cv. 'Liseta' where stimulatory effect of 10^{-4} M SA was observed. Compared to effects on 23 °C, SA treatments did not show prominent inhibitory effect on root elongation, except at highest SA concentration used (10^{-4} M). These findings indicate that SA effects on root initiation and root elongation differ at 23 °C and 35 °C and that moderately elevated temperature possibly changes sensitivity of target root tissues to SA.

Potato is plant species with high basal level of SA, about 100-fold higher than the levels detected in *Arabidopsis* or tobacco (Coquoz *et al.*, 1995). Although most of the SA is present in the bound, physiologically inactive form, SA 2-O-*β*-D-glucoside (SAG), levels of the active, free SA are higher than those found in the leaves of many other plant species (Navarre and Mayo, 2004). Relatively high levels of free SA may be responsible for some differences in potato SA-mediated stress response from *Arabidopsis* or tobacco, such as significant basal levels of SA-inducible *PR-1* present in potato plants in the absence of stress (Navarre and Mayo, 2004). Regardless of high level of endogenous SA, potato thermotolerance can be promoted by exogenously applied SA. Pretreatment with 10^{-5}M SA increased survival rates of five potato genotypes during prolonged antiviral thermotherapy at 42 °C (López-Delgado *et al.*,

2004), while both pretreatment and continuous treatment with low concentrations (10^{-6}-10^{-5} M) of SA analogue acetylsalicylic acid (ASA) improved heat tolerance of potato explants grown *in vitro* at 35 °C (López-Delgado *et al.*, 1998). Results of this study suggest that SA in a range of concentrations from 10^{-6}M to10^{-4}M enhances potato basal thermotolerance during long-term heat stress at 35°C. Both pretreatments and treatments with SA caused increase in the percent of plants developed under 35 °C-HS conditions, with the exception of cultivar 'Marabel' which did not respond on SA when continuously applied. These findings indicate certain variation in genotypes sensitivity to SA during heat stress.

5.4. Effects of SA on HSP Expression

HSPs are molecular chaperones which play important roles in plant heat tolerance by promoting proper folding of heat-denatured proteins, or by forming complexes with denatured proteins to protect them from irreversible thermal aggregation (Lee and Vierling, 2000; Basha *et al.*, 2004). In the present study on potato, effect of SA on the accumulation of heat inducible sHSPs, cytosolic HSP17.6 CI and chloroplast HSP21, and constitutively expressed, but heat-up regulated, cytosolic HSP101 were examined.

SA pretreatment had stimulatory effect on HSP17.6 accumulation at both 35 °C and 45 °C in four potato genotypes. The strongest induction of HSP17.6 accumulation was detected at 10^{-5} M SA for most of the genotypes/cultivars. So far, SA effects on expression and accumulation of this sHSP were examined only in *A. thaliana* (Clarke *et al.*, 2004). Wild-type (Col-0) and SA-overproducing *Arabidopsis* mutants (*cpr5* and *cpr1*) were found to possess higher HSP17.6 levels under heat stress compared to mutants impaired in SA-accumulation (*NahG*). Results of the present study are in concordance with findings on *Arabidopsis* about potential SA role in HSP17.6 CI accumulation, suggesting that exogenous application of SA can also enhance accumulation of this cytosolic sHSP under heat stress.

Interestingly, all applied SA concentrations negatively affected accumulation of chloroplast HSP21 in four potato genotypes at both 35 °C and 45 °C. Reduction in HSP21 accumulation was so strong in leaves of cv. 'Marabel' at 35 °C, that HSP21 was

not detectable in 10^{-6} M and 10^{-5} M SA-pretreatment. Literature data considering SA effect on HSP21 expression are scarce. Thus far, increased levels of chloroplast HSP21 protein were detected in SA-treated leaves of grapevine plants during recovery period after 43 °C-heat treatment (Wang *et al.*, 2010). Results of the present study indicate that exogenous application of SA in the range of 10^{-6}M - 10^{-4}M reduces accumulation of this sHSP in potato.

Relatively low SA concentration (10^{-6} M) promoted accumulation of HSP101 in potato, moderate (10^{-5} M) did not affected abundance, while high concentration (10^{-4} M) reduced accumulation of this protein under 45 °C-heat treatment. At 35 °C, HSP101 accumulation was mainly enhanced by 10^{-5} M SA, while 10^{-4} M-SA pretreatment reduced accumulation of this protein. Interestingly, SA pretreatments also affected constitutive expression of HSP101 at room temperature (23 °C); reduction in HSP101 abundance was detected in most potato genotypes after 10^{-6} M and 10^{-5} M SA pretreatment, while this HSP was not detectable in any of investigated genotypes when 10^{-4} M SA was applied. Effects of SA on HSP101 expression and accumulation were investigated in SA-overproducing *Arabidopsis* mutants (*cpr5* and *cpr1*) and mutants impaired in SA-accumulation (*NahG*) (Clarke *et al.*, 2004). Interestingly, all examined mutants were accumulating similar quantities of HSP101, indicating that differences in endogenous SA levels does not affect abundance of this protein. Results of present study suggest that exogenously applied SA, depending on concentration, may have opposite effect on accumulation of HSP101 in potato under HS. Also, SA reduces HSP101 abundance at 23 °C in range of concentrations from 10^{-6} M to10^{-4} M.

Presented findings of SA effects on HSP accumulation indicate significant differences in SA-mediated regulation of HSP17.6, HSP21 and HSP101 expression under HS. In plants, HSP expression is regulated by a large number of HSFs that cooperate in complex regulatory network. In heat-stressed *S. lycopersicum* seedlings, SA pretreatment potentiated expression of *HSFA1*, *HSFA2*, and *HSFB1*, HSF–DNA binding, as well as HSP70 transcription (Snyman and Cronje, 2008). These findings suggest SA role in modulation of the HSFs expression and activity, which may potentiate HSP expression. However, HSF regulatory networks were investigated mainly in *A. thaliana* and *S. lycopersicum* and there are no data about potato.

5.5. SA Effect on Cu/ZnSOD Expression

All three major types of SOD, including FeSOD (iron cofactor), MnSOD (manganese cofactor) and Cu/ZnSOD (copper and zinc as cofactors), are found in potato microplants (Momčilović *et al.*, 2014). However, Cu/ZnSOD represents the most abundant type of SOD, which determines total SOD activity in the cell. Therefore, it was interesting to investigate effect of SA on the expression of this major SOD type in *S. tuberosum*.

Pretreatment with 10^{-5} M SA had strong stimulatory effect on Cu/ZnSOD accumulation at 23 °C. This is not surprising, since exogenously applied SA has impact on the expression and activity of SOD even in the absence of stress (Rao *et al.*, 1997). According to Miura and Tada (2014), SA inhibits catalase, APX and carbonic anhydrase and this way induces an increase in the ROS levels. In return, ROS act as secondary signal molecules to enhance the activities of antioxidant enzymes, including superoxide dismutase (SOD). Stimulatory effect of 10^{-5} M SA was accentuated at 35 °C, where SA-treated plants accumulated from 3.61- to 26.39-fold higher amounts of Cu/ZnSOD in leaves than SA-non-treated plants. Interestingly, 10^{-5} M SA pretreatment slightly increase abundance of this protein at 45 °C in most potato genotypes. Other SA pretreatments (10^{-6} M and 10^{-4} M SA) did not cause substantial increase in Cu/ZnSOD levels at 23 °C, 35 °C or 45 °C. These results indicate that SA stimulatory effect on Cu/ZnSOD accumulation in potato depend on both temperature treatment and SA concentration.

5.6. HSP 17.6 Expression at Different Time Points After Cessation of SA Treatment

Comparison of the effects of SA pretreatment and SA treatment in potato grown *in vitro* have revealed that continuous SA application has stimulating effect on more of the investigated morphological potato parameters in both HS treatments. Based on this observation, it was reasonable to assume that SA affects potato heat tolerance for limited time after application. Since potato heat tolerance correlates with level of sHSP accumulation (Savić *et al.*, 2012) and SA stimulates HSP17.6 accumulation under HS,

HSP17.6 expression was investigated at different time points after cessation of 10^{-5} M SA application. Particular SA concentration has been selected and used because it induced the highest HSP17.6 accumulation under 45°C-HS.

In leaves of three cultivars, 'Arnova', 'Liseta' and 'Cleopatra', the levels of accumulated HSP17.6 under HS were higher in SA-pretreated than in control microplants at all time points. Interestingly, similar amounts of HSP17.6 were produced and accumulated in shoots of SA-pretreated and control plantlets of 'Marabel' under HS. This cultivar was generally less responsive to both SA treatments and SA pretreatments considering percent of developed plants, internode initiation and internode elongation during 35 °C-HS and 45 °C-HS recovery period compared to 'Arnova', 'Liseta' and 'Cleopatra'. These findings regarding 'Marabel' indicate genotype-specific sensitivity to SA.

Stimulatory effect of SA on HSP17.6 accumulation in 'Arnova', 'Liseta' and 'Cleopatra' was observable during investigated period of 14 days. However, differences between the level of accumulated HSP17.6 in shoots of SA-pretreated and control plantlets were reaching the lowest level 14 days after treatment indicating that SA stimulatory effect is weakening over the time.

6. CONCLUSIONS

6. CONCLUSIONS

- *In vitro* cultures of potato cultivars: ‘Arnova’, ‘Liseta’, ‘Marabel’ and ‘Cleopatra’ were successfully established and plantlets were propagated *in vitro* by single-node stem cuttings (SNC).

- Experimental procedure based on SNC short exposure to excessively elevated temperature (45 °C, 6 h; short-term HS) or prolonged exposure to moderately elevated temperature (35 °C, 20 days; long-term HS) was established and used to examine effects of SA and heat stress on development and growth of potato plants *in vitro*.

- SA in a range of concentrations from 10^{-6} M to10^{-4} M did not affect development of potato plants from SNC at standard growing temperature (23 °C). Similar percentage of plants developed from SNC at 23 °C and during recovery period after exposure to short-term HS indicate that short-term HS was not severe enough to significantly affect development of shoots from axillary buds. On the other hand, long-term HS substantially reduced the number of plantlets developed from SNC. Obtained results indicate that in potato long-term moderately elevated temperatures can cause more devastating effects then short-term excessively elevated ones.

- SA in a range of concentrations from 10^{-6} M to10^{-4} M stimulated development of plants from SNC under long-term HS *in vitro* (35 °C, 20 days) in all investigated potato cultivars, with the exception of cultivar ‘Marabel’ which did not respond on SA treatment. These findings indicate that SA may enhance basal thermotolerance in potato cultivars/genotypes, as well as variation in genotypes´ sensitivity to SA.

- Exogenously applied SA affected shoot elongation *in vitro* at standard growing temperature (23 °C) in potato plants of all investigated cultivars. The effects were concentration-dependent: lower SA concentrations (10^{-6} M - 10^{-5} M) have stimulatory effect on process of internode initiation, while relatively high concentration (10^{-4} M) inhibits this process in most potato cultivars. Internode initiation and internode elongation were showing different sensitivity regarding SA. Internode elongation was affected in all examined cultivars only during continuous 10^{-4} M SA application.

- SA pretreatments, in the range of concentrations from 10^{-6} M to 10^{-4} M, do not affect internode initiation and elongation either under standard or elevated temperatures, while SA treatments in the same range of the concentrations stimulate these processes under both short-term and long-term HS.

- Both SA pretreatments and SA treatments, in the range of concentrations from 10^{-6} M to 10^{-4} M, strongly inhibit root initiation and elongation at standard growth temperature and after short-term HS, while SA treatments do not show prominent inhibitory effect on root elongation in the same range of the concentrations under long-term HS. The results suggest presence of interaction between SA and HS in the process of root elongation, possibly depending on duration and intensity of HS, as well as on duration of exposure to SA.

- SA pretreatment had stimulatory effect on HSP17.6 accumulation at both 35 °C and 45 °C in four potato genotypes. The strongest induction of HSP17.6 accumulation was detected at 10^{-5} M SA for most of the genotypes/cultivars. Interestingly, all applied SA concentrations inhibited accumulation of chloroplast HSP21 in four potato genotypes at both 35 °C and 45 °C. Relatively low SA concentration (10^{-6} M) promoted accumulation of HSP101 in potato, moderate (10^{-5} M) did not affected abundance, while high concentration (10^{-4} M) reduced accumulation of this protein under 45 °C-heat treatment. At 35 °C, HSP101 accumulation was mainly enhanced by 10^{-5} M SA, while 10^{-4} M-SA

pretreatment reduced accumulation of this protein. These findings of SA effects on HSP accumulation indicate significant differences in SA-mediated regulation of HSP17.6, HSP21 and HSP101 expression under HS.

- Pretreatment with 10^{-5} M SA only had strong stimulatory effect on Cu/ZnSOD accumulation at 23 °C in leaves of investigated potato cultivars. Stimulatory effect of 10^{-5} M SA was accentuated at 35 °C; SA-treated plants accumulated significantly higher amounts of Cu/ZnSOD than SA-non-treated plants. Interestingly, 10^{-5} M SA pretreatment caused slight increase in abundance of this protein at 45 °C in most potato genotypes. These findings indicate that stimulatory effect of SA on Cu/ZnSOD accumulation in potato depends on both temperature treatment and SA concentration.

- Stimulatory effect of SA on HSP17.6 accumulation in 'Arnova', 'Liseta' and 'Cleopatra' was observable during investigated period of 14 days. However, differences between the level of accumulated HSP17.6 in shoots of SA-pretreated and control plantlets were reaching the lowest level 14 days after treatment indicating that SA stimulatory effect is weakening over the time. Interestingly, similar amounts of HSP17.6 were accumulated in shoots of SA-pretreated and control plantlets of 'Marabel' under HS. This cultivar was generally less responsive to both SA treatments and SA pretreatments considering percent of developed plants, internode initiation and internode elongation during both short-term and long-term HS compared to 'Arnova', 'Liseta' and 'Cleopatra'. These findings indicate variation in sensitivity to SA between cultivars.

7. REFERENCES

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Прилог 1.

Изјава о ауторству

Потписани-а Masoud M. A. Hfidan

број уписа

Изјављујем

да је докторска дисертација под насловом

Effect of salicylic acid on the expression of heat-shock proteins and morphology of potato (*Solanum tuberosum* L.) plants under heat stress *in vitro*

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 1.9.2014.

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора _____Masoud M. A. Hfidan_____

Број уписа _____

Студијски програм _____Физиологија и молекуларна биологија биљака_____

Наслов рада __Effect of salicylic acid on the expression of heat-shock proteins and morphology of potato (*Solanum tuberosum* L.) plants under heat stress *in vitro*___

Ментор _____др Ивана Момчиловић и др Ивана Драгићевић_____

Потписани _____Masoud M. A. Hfidan_____

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, _____1.9.2014._____ _____

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Effect of salicylic acid on the expression of heat-shock proteins and morphology of potato (*Solanum tuberosum* L.) plants under heat stress *in vitro*

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, 1.9.2014. _____

1. Ауторство - Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце, чак и у комерцијалне сврхе. Ово је најслободнија од свих лиценци.

2. Ауторство – некомерцијално. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела.

3. Ауторство - некомерцијално – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела. У односу на све остале лиценце, овом лиценцом се ограничава највећи обим права коришћења дела.

4. Ауторство - некомерцијално – делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца не дозвољава комерцијалну употребу дела и прерада.

5. Ауторство – без прераде. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца дозвољава комерцијалну употребу дела.

6. Ауторство - делити под истим условима. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.