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EFFECT OF THERMOTHERAPY IN COMBINATION WITH MERİSTEM CULTURE FOR ELİMİNATİNG *POTATO VIRUS Y* (PVY) AND *POTATO VIRUS S* (PVS) FROM INFECTED SEED STOCKS

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ABSTRACT

The present study was conducted to investigate the impact of thermotherapy along with meristem culture for eliminating *Potato virus Y* (PVY) and *Potato virus S* (PVS). The plantlets were subjected to enzyme linked immunosorbent assay (ELISA) to check the presence of targeted viruses. A total of 110 plants including 65 infected with PVY and 45 with PVS belonging to six potato cultivars (Agria, Russet Burbank, Shepody, Granola, Marabel, and Sante) were subjected to thermotherapy (under constant temperature of 36°C and constant light conditions) for a period of five weeks. The obtained results revealed no PVY and PVS positive plants after five week of thermotherapy. However, plant mortality was observed in cv. Granola, R. Burbank, Shepody and Agria. Cv. Granola was sensitive, whereas, Sante and Marabel were found to be resistant cultivars. Marabel and Sante responded effectively to thermotherapy treatment by producing PVY and PVS negative plants with no plant mortality after application of thermotherapy. The study showed that propagation of clean production material is possible by applying thermotherapy treatment along with tissue culture method by using meristem culture and shoot tips cultures.

Keywords: Potato, thermotherapy, PVY, PVS, DAS-ELISA, Meristem culture.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a tuber crop and ranked as fourth important food crop after wheat, rice and maize worldwide (Jones *et al*., 1994). It is among the cheapest source of vitamins, carbohydrates, proteins and minerals (Ali *et al*., 2013). Turkey is among the potential potato producing countries, but yield is still low as compared to other countries including Europe which are harvesting 40-100 ton/ha (Çalikan *et al*., 2010). Potato is a vegetatively-propagated plant, and is affected by number of fungal, bacterial and viral diseases that are easily transmitted by the infected tubers from one generation to the next and one region to another by causing considerable quality and yield losses (Truta, 1997). Viruses are responsible for degeneration and poor plant growth and vigor leading to reduction in the resistance against the diseases and productivity of potato cultivars (Sangar *et al*., 1988). Viral diseases are considered as major limiting factors for potato production and its deterioration (Al-Shahwan *et al*., 1998; Hamm and Hane, 1999; Hord and Rivera, 1998; Jan and Mohammad, 1994; Mansour, 1999; Omer and El, hassan, 1992).

Potato virus Y (PVY) and *Potato virus S* (PVS) are among the important viruses causing severe infection to plants health that results in poor production (Awan *et al*., 2005). The PVY induced symptoms vary from almost indistinguishable mosaic patterns and necrosis leading towards premature death of plants. The sympotoms mainly differ based on virulent viral strain and infected cultivar (Silberschmidt and Kramer, 1942). PVY is the most important viral pathogen of potato that can cause yield loss of 10-100% (Tsedaley, 2015). PVS occurs globally in potato cultivated fields and is capable to reduce yield loss upto 10% or result in high infection of seed tubers (Struik and Wiersema, 1999). These two viruses are mechanically transmittable; require regular removal of infected seed and monitoring in order to maintain the yield potential of the cultivars. Therefore, increasing demands of good quality of virus-free potato tubers and seed is among the important challenges to meet considering cost economic ratio in potato production (Awan *et al*., 2005).

The plant viruses can be successfully eliminated by knowing the mechanism of mode of replication and of virus within the plant (Awan *et al*., 2005). Furthermore,the elimination methods are virus and cultivar sensitive (Panattoni *et al*., 2013). There are various methods including tissue culture, chemotherapy and thermotherapy that have been used for the purpose to eliminate the virus from the infected tubers seed and plantlets (Mahmoud *et al*., 2009; Dhital *et al*., 2008). Subsequently, heat therapy has been successfully tested for the elimination of plant pathogens worldwide. However, the mechanism of heat therapy on viruses is not

well understood. This method is believed as effective methods that can inhibit synthesis of proteins by blocking their transcription and viral replication (Mink, *et al*., 1998). In plant thermotherapy, exposing the virus infected explants at a temperature between 35 to 40°C for 14 to 28 days is suggested as effective method for the elimination of virus (Agrios, 2005). Increasing thermotherapy duration could be productive for the elimination of virus; however, it can affect the regeneration and survival of treated plantlets (Tan *et al*., 2010). In meristem culture, virus elimination success percentage is more oftenly is affected by size of the excised meristems, tested cultivar including virus and plant species (Loebenstein *et al*., 2001). In the apical meristem, ablation of the selected cells can increase the persistence of meri-clone regeneration, which results in the production of virus-free plantlets at high propotion (Nascimento *et al*., 2003; Wasswa *et al*., 2010; Wang and Valkonen, 2008). According to Wang and Valkonen (2008), this procedure alone is difficult and resulting in lowering the virus elimination efficiency meristem tip culture combined with thermotherapy due to its ease, safety for generating virus-free plants and its apparent affordability has been successfully applied in various commercial crops. *In vitro* thermotherapy followed by meristem culture considerably can increase the virus elimination by permitting a very limited number of specialized cells to carrying minimum infective virus (Panattoni*et al*., 2013).The perfect results about virus eradiation from the propagative material are obtained by the combination of thermotherapyin combination with meristem culture; where alone meristem culture fails to produce successful results (Waswa *et al*., 2017).

PVY and PVS are considered as the potential viruses effecting potato production in Turkey (Bostan and Haliloglu, 2004). There is approximatelydemand of 450,000 t potato seeds per year (Anonymous, 2018). Despite of huge demand, there is no state agenices for providing virus free seeds to the farmers in Turkey. However, seeds are being imported, multiplied using tissue culture techniques and later distributed to the growers by using the distribution channel of private companies. (Bostan and Demirel, 2004).

The application of meristem-tip culture in combination with virus elimination techniques can help in producing virus free plant. Keeping in view the importance of above discussed prospecitve,the present study was conducted to study the impact of thermotherapy duration on potato plantlet survial and virus elimination efficiency in tested potato cultivars.

MATERIALS AND METHODS

The experiments were conducted at the Laboratory of Tissue Culture and Molecular Biology, Toros Agri, Biotechnology Center, Adana, Turkey.

Potato cultivars: Six potato cultivars (Russet burbank, Agria, Shepody, Granola, Sante and Marabel) were obtained from potato producers of the Adana and Sivas province.

Production and selection of virus-infected stocks: A total 720 tubers of six potato cultivars were grown in plastics pots and kept in the plant growth chamber (24 ± 2 °C and 70 ± 5% RH with a 16:8 h light: dark photoperiod) under controlled conditions. After plant emergence at the four leaf stage, the leaves samples were collected and subjected to double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) by treating with monoclonal and polyclonal antisera kit according to the protocol defined by manufacturer (Bioreba AG, Switzerland) for the indexing of PVY and PVS.

The thermotherapy efficiency experiment: Thermotherapy application at 36 °C temperature with 24 h of light (light intensity above 10,000 lux) was applied on selected virus positive plants for period of 6 weeks in a thermotherapy chamber. During the 3rd and 5th week and after the 1st, 3rd and 6thweek of thermotherapy, the plants were kept under control conditions and subjected to DAS-ELISA according to manufacturer instruction (Bioreba AG, Switzerland) to check the impact of thermotherapy on virus infected plant samples. The plants after6th week of thermotherapy were used for *in vitro* studies.

Production of *in vitro* plantlets: The selected terminal buds were excised from mother plants to promote axillary bud growth and introduced in to the *in vitro* studies. The excised plants having 5 to 8 cm length were selected for surface sterilization by soaking it in the water solution having liquid detergent for 30 minand then socked in 70% ethanol (EtOH; C_2H_6O)till 5. Later were soaked in liquid solution of 17% of 5%commercial sodium hypochlorite (NaClO) having 1-2 drops of Tween-20 for the period of 3 minute. The immature leaves, pedicel, and the outer leaf primordiasurrounding the meristem region were discarded and apical meristem (~0.1 to 0.5 mm) were excised from plants with the help of sharp microsurgical blades with fine pointed needles using a 100× magnification using microscope in sterile laminar flow cabin.

The excised meristems were cultured using 7 ml of MS medium (Murashige and Skoog, 1962)including potato growing mediain the glass tubes (15 x 2.5 cm). Meristem growing media included 4.3 g MS medium, 30 g saccharose, 100 mg inositol, 1 mg MS vitamin (for 100 ml; 0.01 g Thiamine HCl, 0.05 g PyrodoxineHCl, 0.05 g Nicotinic acid), 0.1 mg GA3, 0.4 mg kinetin, 0.5 mg IAA and 7 g agar in 1000 ml of distilled water having pH 6. The explants were maintained in the growth chamber (24 ± 1 °C and 70 ± 5% RH with a 16:8 h light:dark

photoperiod having light intensity of 4000 lux under controlled conditions.

The regenerated meristems at a height of 4-6 cm were utilized for the multiplication purpose by nodal cuttings. Each node of a plantlet consisted of 0.2-0.5 cm long stem segments with an axillary bud and its minor leaf that was cut and subcultured to obtain meristem clones (Wang *et al*., 2006).The meri-clones were subjected to DAS-ELISA (according to above describe protocol) to check the presence of virus-infection at 3rd and 7th subculture stage.

ELISA indexing on plants maintained in greenhouse: The plants obtained from meri-clones were shifted to breeding bed having peat moss and perlite mixture in the greenhouse. The leaf samples were collected and tested with DAS-ELISA (according to above describe protocol) to check the presence of PVY and PVS.

Data collection and analysis: The thermotherapy efficiency in terms of virus elimination percentage was calculated as ratio of number of virus free plants to the total number of cultures. Whereas, percent survival was determined as number of plants that survived per thermotherapy period. The significance among virus and genotype interaction, plant survival and the efficiency of virus elimination were calculated by using analysis of variance (ANOVA) by using Syntactically Awesome style sheet (SASS) software. Means of significant ($P \leq 0.05$) main effect and interactions were compared using Duncan Multiple Range Test (DMRT).

RESULTS

Detection of PVY and PVS Infection: A total of 65 plants, including 45 infected with PVY and 20 infected with PVSwere selected based on DAS-ELISA results obtained during production and selection of virus infected stock. The virus positive plants were used in thermotherapy experiments.

Effect of thermotherapy on accumulative virus infectivity in tested cultivars: The survival of plants during and after heat treatment significantly depended on the type of cultivars and type of virus infection during application of thermotherapy ($P \leq 0.05$). The efficiency of thermotherapy for getting virus free plants after heat treatment was significantly influenced by tested cultivars and interaction between types of virus infection ($P \leq 0.05$). Furthermore, virus and cultivars interaction revealed that different cultivars responded differently to thermotherapy treatment.

The effect of thermotherapy was recorded significantly among different tested cultivars and viruses (Table 1). Shepody responded maximum (df=5; MS=0.561;F=3.023; $P \leq 0.05$, n=10),whereas,Marabel responded minimum (df=5; MS=0.561;F=3.023; $P \leq 0.05$, n=9) virus infected samples during the 3rd week of thermotherapy treatment. Furthermore, Sante (df=5; MS=0.363;F=1.5173; $P \leq 0.05$, n=7) and Marabel responded with minimum (df=5; MS=0.363;F=1.5173; $P \leq 0.05$, n=9), whereas R. Burbank (df=5; MS=0.363;F=1.517; $P \leq 0.05$, n=14), and Shepody responded maximum (df=5; MS=0.363;F=1.517; $P \leq 0.05$, n=10)virus infectivity samples during the 5thweek of thermotherapy. There was statistically similar virus infectivity responses recorded on R. Burbank (df=5; MS=0.561;F=3.023; $P \leq 0.05$, n=14), Agria (df=5; MS=0.561;F=3.023; $P \leq 0.05$, n=13) and Granola (df=5; MS=0.561;F=3.023; $P \leq 0.05$, n=12) during the 3rd week. Additionally similar virus infectivity responses were recorded on Agria (df=5; MS=0.363;F=1.5173; $P \leq 0.05$, n=13) and Granola (df=5; MS=0.363;F=1.5173; $P \leq 0.05$, n=12) during 5thweek of thermotherapy respectively.

Moreover, all the tested cultivars showed virus negative results when analysed after 1st week later finishing the thermotherapy treatment till the end of experiment. The survival rate of Sante and Marabel were recorded maximum during and after thermotherapy treatment during the experiment. Whereas, minimum survival percent was recorded on Agria, Granola, Shepody and R. Burbank respectively until 6th week after thermotherapy (Table 1).

Effect of thermotherapy on PVY elimination and plantlets survival in tested cultivars

***During thermotherapy treatment*:** The effect of thermotherapy and plantlet survival was significantly different among the tested PVY infected genotypes (Table 2). After2nd week of thermotherapy, no PVY infected and dead plants wererecorded in Marbel throughout the experiment. It was revealed that maximum PVY infection was recorded on Shepody during the3rd week of thermotherapy.Whereas, the results revealed that R.Burbank with maximum virus infected samples and Sante and Marabel were recorded PVY negative during the 5th week of thermotherapy.

***Post thermotherapy treatment*:** The results of 1stto 6th week postthermotherapy revealed the total elimination of PVY from the tested plantlets. However, the plant mortality with increased ratio was recorded in a persistent manner in tested cultivars except on cv. Sante and Marabel. Maximum plant mortality was recorded on cv. Granola after 1st week of thermotherapy, followed by 40 and 50 % in Agria post 3rd and 6th week of thermotherapy respectively.

No plant mortality was observed in all tested cultivars, except cv. Granola during 1st week post thermotherapy. Minimum plant mortality was recorded in Shepody during 3rd week post thermotherapy followed by cv. Granola during 6th week post thermotherapy.

Effect of thermotherapy on PVS elimination and plant let survival in tested cultivars

***During thermotherapy treatment*:** Significant difference was noted among the PVS infected plantlets of tested cultivars when exposed to thermotherapy (Table 2). During 3rd week of thermotherapy minimum PVS infected samples were recorded in cv. Sante followed by cv.Marabel. No plantlet of cv. Granola, Sante and Marabel was found infected with PVS during 5th week of thermotherapy.

***Post thermotherapy treatment*:** There was no plantlet found infected with PVS after 1st week -6th week post thermotherapy. However, 25 % plant mortality was recorded in cv. Granola and cv. R.Burbank after 1st week post thermotherapy.

There was no plant mortality observed in cv. Agria, Shepody, Sante and Marabel during the 1st week after thermotherapy. Minimum 25 % and maximum 50 % plant mortality was observed in R. Burbank and Granola during the 3rd and 6th week after thermotherapy respectively.

DAS-ELISA results of *in vitro* and greenhouse production: The plants produced under *in vitro* culture conditions and later used in greenhouse when tested by DAS-ELISA revealed all tested plants as PVY and PVS negative.

Table 1. Effect of thermotherapy on survival and accumulative virus elimaination of meristem derived potato plantlets.

Genotype	3rd week of thermotherapy	5th week of thermotherapy	After 1st week of thermotherapy	After 3rd week of thermotherapy	After 6th week of thermotherapy
R.BURBANK	71.43ab	50.00a	0.00 (07.14)*	(21.43)*	0.00(28.57)*
AGRIA	76.92ab	46.15ab	0.000	(38.46)*	0.00(46.15)*
SHEPODY	90.00a	50.00a	0.000	(10.00)*	0.00(30.00)*
GRANOLA	66.67ab	33.33ab	0.00(16.67)*	(33.33)*	0.00(33.33)*
SANTE	42.86bc	00.00b	00.00	0.00	00.00
MARABEL	11.11c	00.00b	00.00	0.00	00.00

Table 2. Effect of thermotherapy on survival PVY and PVS elimaination from meristem derived potato plantlets.

Cultivar	3rd week of thermotherapy		5th week of thermotherapy		After 1st week thermotherapy		3rd week of after thermotherapy		After 6th week of thermotherapy	
	PVY	PVS	PVY	PVS	PVY	PVS	PVY	PVS	PVY	PVS
R. Burbank	70.00	75.00	60.00	25.00	0.00	0.00 (25)*	0.00 (20)*	0.00 (25)*	0.00 (30)*	0.00(25)*
Agria	80.00	67.00	50.00	33.00	0.00	0.00	0.00 (40)*	0.00 (33)*	0.00 (50)*	0.00 (33)*
Shepody	100,00	67.00	57.00	33.00	0.00	0.00	0.00 (14)*	0.00	0.00 (29)*	0.00 (33)*
Granola	75.00	50.00	50.00	00.00	87 (13)*	0.00 (25)*	0.00 (25)*	0.00 (50)*	0.00 (25)*	0.00 (50)*
Sante	20.00	00.00	00.00	00.00	0.00	0.00	0.00	0.00	00.00	00.00
Marabel	00.00	25.00	00.00	00.00	0.00	0.00	0.00	0.00	00.00	00.00

DİSCUSSİON

This present study was conducted with an aim to examine the effect of thermotherapy in combination with meristem culture for eliminating PVY and PVS infection between the selected potato cultivars. Furthermore, plantlets survival response was genotype dependent post thermotherapy treatment. Thermotherapy along with meristem culture showed appreciable results. It was concluded, that the protocol could act as ahelpfultool in virus eradication programs of potatoes (Brown *et al*. 1988; Faccioli and Marani 1998). It was observed that virus elimination percentage depends upon the duration and intensity of temperature applied during thermotherapy applications (Quak 1977; Waswa *et al*., 2017). Nascimento *et al*. (2003) reported that there was significant eredication of PVY using *in vitro* techniques. Carvalho *et al*. (2002), reported meristem alone as less effective, whereas obtained complete eradicaiton of citrus exocortis pospiviroid (CEVd) and citrus tristeza closterovirus (CTV) by using thermotherapy in combination with meristem culture. It is important to mention that variable results during obtaining virus free plants may be attributed to virus inactivation temperature

(Fernow *et al*., 1962; De Bokx, 1972; Hull, 2002). Quak (1977) reported thermotherapy as an effective treatment at the temperature of 38±2ºC as capable to decrease virus concentration. The *in vitro* generated plant when exposed at temperatures between 35 and 40 °C showed virus negative results (Leonhardt *et al*.,1998). According to Awan *et al*. (2005) high temperature inducts gathering of protein subunits which results in protecting the viral nucleic acid to become weak resulting in apperance of temporal fissures that inactivates the virus and its concentration ultimatly decreased the nucleases activity. Furthermore, the tested cultivar along with virus type has a significant impact on virus elimination percentage as well. The use of meristem culture has some advantages, due to restriction of virus movement and inhibition of replication (Faccioli and Marani 1998). According to Awan *et al*. (2005), meristem culture results in inducing the competition among the rapidly host cells, location of virus particle, protein and nucleic acid synthesis which creates imbalance situation between synthesis and degradation of virus particles resulting ultimatley the inactivation of virus.

During *in vitro* thermotherapy treatment, decrease in the survival of plantlet with the increase in the duration of thermotherapy was also variable depending upon potato cultivar and type of virus infection. It was concluded that plantlet and meristem exposed to thermotherapy for a long period of time can adversely affect metabolic activities of the plant and meristem leading to the plant death (Ali *et al*., 2013).

The ELISA technique has played a contributed role in the diagnosis of virus due to its simple methodology and less time requirement to get results (Torrance and Jones, 1981).Now a days, ELISA technique is widely used for the detection of viruses in crops (Boonham *et al*., 2014). Being cost effective, scalable, robust and easy to use in order to test samples in large quantity in short period of time, this technique has various advantages (Torrance and Jones, 1981); However have various drawbacks that limitates its use as a universally applicable test for plant virus diagnosis. Moreover, DAS-ELISA has been also used as routine test inquarantine laboratories of many countries included Turkey for indexing potato viruses. However, it is important to mention the nucleic acid based molecular techniques such as nucleic acid spot hybridisation (NASH) and Polymerase Chain Reaction (PCR) have revolutionized the area of virus detection due to providing authentic and clear results, which scientists have failed to produce using ELISA technique (Boonham*et al*., 2014). Furthermore, each methodology has its advantages and limitation but there is needed to design a comprehensive virus management and detection technique. The mentioned serological and molecular detection techniques in combination with virus elimination technique could be helpful for the effective virus management program and continuing a safe and sustainable agriculture system.

Conclusion: It was concluded that virus elimination efficiency was affected by potato cultivar, virus type on one hand and virus type along with thermotherapy duration interactions on the other. Furthermore, the plantlet survival response based on cultivar type was also influenced by thermotherapy. Sante and Marabel were showed effective virus elimination and best survival in thermotherapy. Thus are very helpful for potato producer having virus-free potato stocks by using thermotherapy following the *in vitro* culture.

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