INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 2, p. 49-57, 2017

RESEARCH PAPER OPEN ACCESS

Effect of sodium hypochlorite on *in vitro* sterilization of six promising potato cultivars

Abdul Ahad Qureshi[1], Nadeem Akhtar Abbasi[*2], Ishfaq Ahmad Hafiz[2], Muhammad Kausar Nawaz Shah[3]

[1]*PMAS Arid Agriculture University Rawalpindi, Sub-Campus Khushab, Khushab, Pakistan*

[2]*Department of Horticulture, PMAS Arid Agriculture University, Rawalpindi, Pakistan*

[3]*Department of Plant Breeding and Genetics, PMAS Arid Agriculture University, Rawalpindi, Pakistan*

Key words: Sodium hypochlorite, Sterilization, *In vitro*, Potato

http://dx.doi.org/10.12692/ijb/11.2.49-57 Article published on August 20, 2017

Abstract

In vitro establishment of pathogen free, healthy germplasm for commercial and research purpose need asepsis. Each plant and type of explant has different protocol for disinfection. Excess amount of these sterilant can cause toxic affect. Therefore, this study was conducted to investigate the effect of different concentrations (0, 5, 10 and 15 %) and time duration (5, 10 and 15 minutes) of Sodium hypochlorite (NaOCl) on *in vitro* sterilization of potato explant to find out best sterilant dose with least toxic effect on *in vitro* culture establishment. The study shows that 10% NaOCl for 15 minutes proved significant sterilant while keeping low toxicity. It gave maximum number of healthy plants in each variety as well as minimum quantity of infected and non-growing culture with 92.2% healthy, 3.3% infected and 4.5% non-growing cultures for Kuroda, 91% healthy, 4.5% infected and 4.5% non-growing cultures for Astrex, 87.7% healthy, 5.6% infected and 6.7% non-growing cultures for Lady Rosetta, 92.2% healthy, 4.5% infected and 3.3% non-growing cultures for Sante, 94.5% healthy, 3.3% infected and 2.2% non-growing cultures for Diamant and 93.3% healthy, 4.5% infected and 2.2% non-growing cultures for Hermes. While higher doses eliminate the contaminants, but endure toxic effects on explant.

* **Corresponding Author:** Nadeem Akhtar Abbasi ✉ nadeemabbasi65@yahoo.com

Introduction

Potato (*Solanum tuberosum* L.) is the world's most popular and nutritious vegetable crop (Fernie and Willmitzer, 2001; FAO, 2008). It produces more food per unit area in an efficient time on less land and in mild climates than any other food crop. It is the fourth most important crop by volume of production in Pakistan, cultivated over an area of 174.4 thousand hectares with an annual production of 3802.2 thousand tones (t), yielding 21.8 tones ha^{-1} (FAO, 2014). Potato is usually propagated asexually by means of tubers. However, *in vitro* propagation of potato can be done from different explants on MS medium to get premium quality pathogen free planting material (Hossain, 1994; Rabbani *et al*., 2001; Zaman *et al*., 2001).

Micro-propagation is a tool which allows rapid and mass production of genetically identical plants in a limited space, inputs and time (Odutayo *et al.,* 2004). Tissue culture technique involves various steps i.e. selection of explant, its sterilization and establishment, shoot proliferation and production of micro tubers. Other than growth hormones, temperature, relative humidity and photoperiod of the growing culture can also effect the growth and development of plantlets (Hussey and Stacey, 1981).

First step for a successful culture establishment is asepsis. Maintenance of aseptic or sterile conditions are the basic needs for a success story. It depends on the removal of exogenous and endogenous contaminating microorganisms (Constantine, 1986; Buckley and Reed, 1994). Contaminated plants can reduce multiplication and rooting rates or may die.

It is necessary to remove foreign contaminants including bacteria and fungi from explants. To eliminate contamination during *in vitro* propagation different methods have been developed (Barrett and Casselles, 1994; Hussain *et al.,* 1994; Herman, 1996). To maintain this condition, all instruments, jars or tubes, media as well as explant itself must be sterilized and all operation should be carried out in laminar airflow cabinet (Chawla, 2003).

The surfaces of living plant materials are naturally contaminated with microorganisms from the environment, so surface sterilization of explants in chemical solutions is a critical preparation step.

The disinfectants usually used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate. Most laboratories use sodium or calcium hypochlorite or various commercial bleaches for surface sterilization of explants. Since these sterilizing agents are toxic to the plant tissue, contamination must be removed without killing the plant cells. Microbes in these plant cultures increase culture mortality.

Different infections can hinder culture growth, tissue necrosis, shoot and root proliferation rate. Although tissue culture techniques usually involve growing stock plants in a way that will minimize infection. Treating the plant material with disinfecting chemicals and sterilizing the tools also help to kill superficial microbes (George, 1993). To control contaminations in plant tissue cultures, there are three main issues: preventing the introduction of microorganisms with the initial plant material, preventing their introduction from the environment during sub-culturing and reducing microbial contamination in the cultures at the stage of multiplication and rooting.

The most effective way of preventing microbial contamination in tissue culture is to eliminate them from the initial explants. The methods for reducing contaminations include the use of explant of donor plants under a strict hygienic condition, efficient sterilization of the initial explants and reducing the size of initial explants just to apical meristem.

The procedures for sterilization are various, depending on plant species and explant. Each plant material has variable surface contaminant levels, depending on the growth environment, age and explant used for micro-propagation. It is difficult to determine standard sterilization procedure that apply to all plants.

Therefore, the present study was aimed at standardize the sterilization method of explants for six promising potato cultivars i.e. Kuroda, Asterix, Lady Rosetta, Sante, Diamant and Hermes for micropropagation, using sterilizing agent (Sodium Hypochlorite) by varying its concentration and duration of exposure.

Material and methods

The present study was carried out at Plant Tissue Culture Laboratory, Department of Horticulture, PMAS Arid Agriculture University Rawalpindi, with the objective to evaluate the effect of different concentrations and exposure time of sodium hypochlorite on explants of six potato cultivars for *in vitro* culture establishment. These six cultivars of potato are the most prominent varieties grown by Pakistani farmers. Three are the red skinned i.e., Kuroda, Asterix and Lady Rosetta and others are the white skinned i.e., Sante, Diamant and Hermes.

Preparation of explant

For obtaining sprouts, the tubers were washed thoroughly with water and treated with fungicide named Mancozeb for 4-5 minutes and sown in sand filled pots under glasshouse condition. The sprouts were ready after 15 days of growth. Sprouts of about 1-2 cm were collected from mother plant of these varieties in water filled beaker from field and brought to laboratory and kept under running water for one hour, prior to sterilization in laminar airflow cabinet.

Execution of disinfection treatments

All explants were incubated in 70% ethanol for 1 min and then transferred to the sterilization solution. A group of control explants were rinsed with 70% ethanol and incubated in sterilized, autoclaved water. For surface sterilization, 5%, 10%, and 15%, solutions of NaOCl (SIGMA-ALDRICH, USA reagent grade NaOCl with active chlorine 10-15 %) were prepared. Explants were incubated for 5, 10 and 15 minutes in each concentration of sterilizing agents. For better contact of sterilizing agent with explant, few drops of Tween 20 detergent were added while disinfecting. After decontamination treatments, all explants were rinsed three times with sterilized water.

All instruments and glass wares used in this experiment were also autoclaved at 15 psi for 45 minutes. To evaluate the response of different concentration and exposure time of sodium hypochlorite, explants were cultured in MS medium with vitamins, sucrose (30 gm l^{-1}), and agar (7 gm l^{-1}). After treatment, the cultures were placed in growth room having 25 ^{o}C temperature, 16 h photoperiod and 3500 lux of light intensity. The observations were recorded regularly for 30 days to record non-growing, infected and healthy cultures.

Statistical analysis

Thirty explants were used in each sterilization treatment and each treatment was replicated three times. Data were analyzed by using three factor factorial design, including varieties, concentration of NaOCl and exposure time of explant. The effect of various treatments was assessed by analysis of variance (ANOVA) for healthy, infected and non-growing plants percentage. The mean values were compared using the Tukey's HSD test. The differences between treatments were considered significant at $p \leq 0.05$ and designated by different letters. All statistical analyses were done with Statistix 8.1 (Analytical software, USA).

Results

Effect of sodium hypochlorite on in vitro infection control

Sodium hypochlorite concentrations significantly affected plant sterilization (%), when applied for different durations. All the plants in the control group 0% NaOCl with 5, 10 and 15 minute exposure time were contaminated after a few days, giving 94 to 100 % contamination in all potato cultivars (Fig. 1.). After fifteen days, plants in 5% NaOCl for 5 minutes showed 84 to 87% contamination followed by 5% NaOCl for 10 minutes (60%) and so on. Trend shows that as we increased the concentration and time duration infection percentage decreased gradually. Interaction of concentration and exposure timing exhibits significant difference among the treatments. However, 10% NaOCl for 10 min and 15% NaOCl for 5 min were statistically at par.

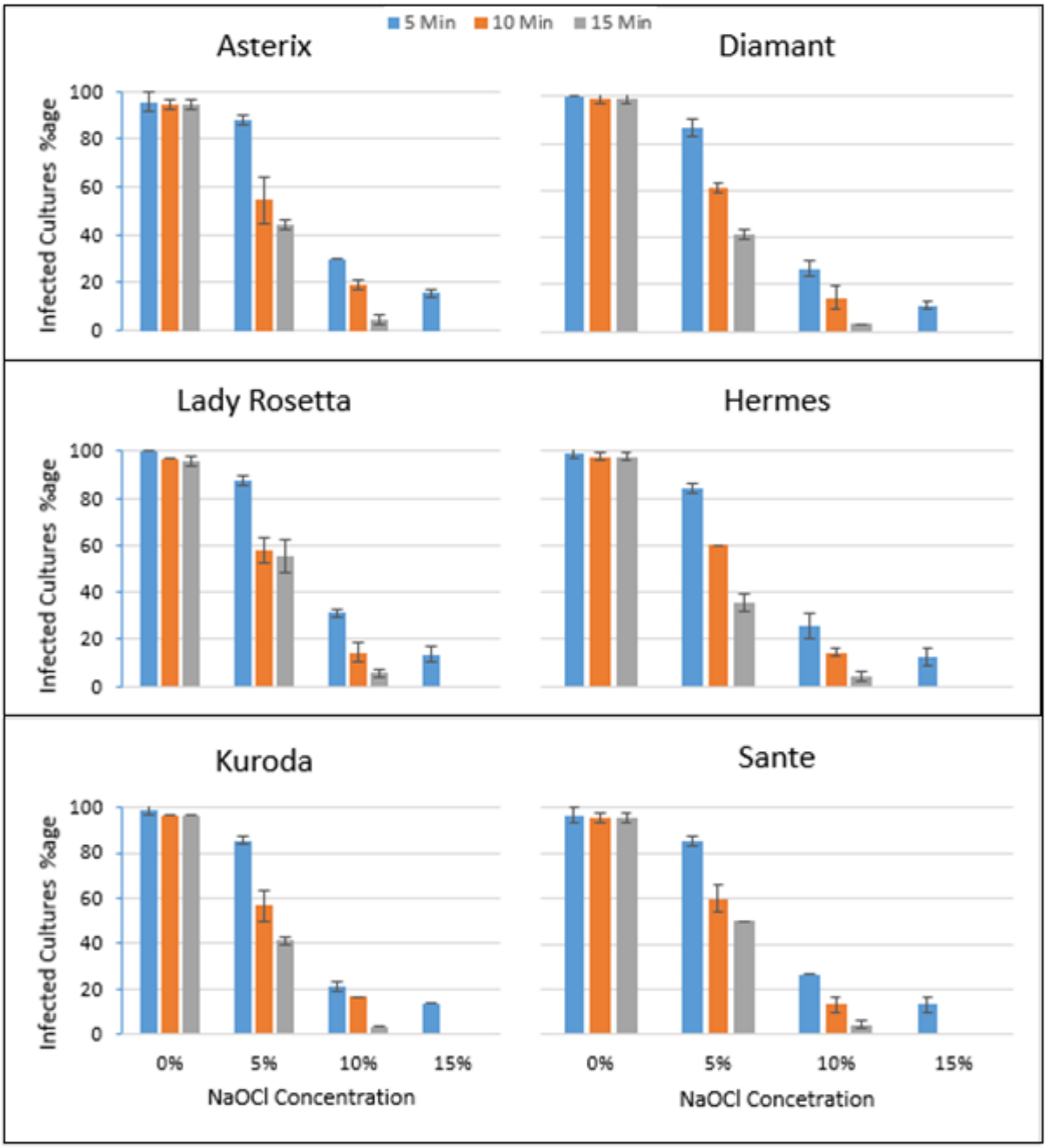

Fig. 1. Variety wise infected cultures percentage of potato explants as affected by concentration and time duration of NaOCl.

Effect of sodium hypochlorite on in vitro plant mortality

It is obvious from the data (Fig. 2.) that increasing the concentration and dipping duration increased the mortality rate significantly and was recorded higher in 15% NaOCl for 15 minutes (56-62%) followed by 10 minutes (38-48%) and 5 minutes (16-21%) dipping with same concentration While, lowest mortality rate (0%) was observed in 0 and 5 % NaOCl concentration respectively (Fig. 2.).

Effect of sodium hypochlorite on in vitro healthy cultures establishment

Concentrations of NaOCl employed in sterilization differed significantly ($p \leq 0.05$). The mean values of different concentrations of NaOCl (Fig. 3.) show that the effectiveness of the sterilization procedure increases with concentration and exposure timing. 10 % NaOCl for 15 minutes dips treatments had highest mean of healthy explants free from contamination i.e. 94.44% in Diamant, 93.33% in Hermes, 92.22% in

Sante and Kuroda, 91.11% in Asterix and 87.77% in Lady Rosetta on the 30th day after culture establishment. The data indicate (Fig. 3.) that with the increase in concentration and duration of NaOCl survival rate of healthy plants was also increased. The overall best percentage of healthy plants was obtained by 10% NaOCl for 15 minutes (87-94%) followed by 10 minutes (73-82%), 5 minutes (63-72%) with same concentration, and 62-72% was with 15% NaOCl for 5 minutes, while other treatments showed less than 60% of healthy culture establishment. The poorest performance was shown by group of control (0% NaOCl with 5, 10 and 15 minutes duration). However, the concentrations had no significant effect among varieties except Diamant (Fig. 3.). Sterilization show an increasing trend as we increase the concentration of sodium hypochlorite up to 10% (v/v) for 15 minutes duration.

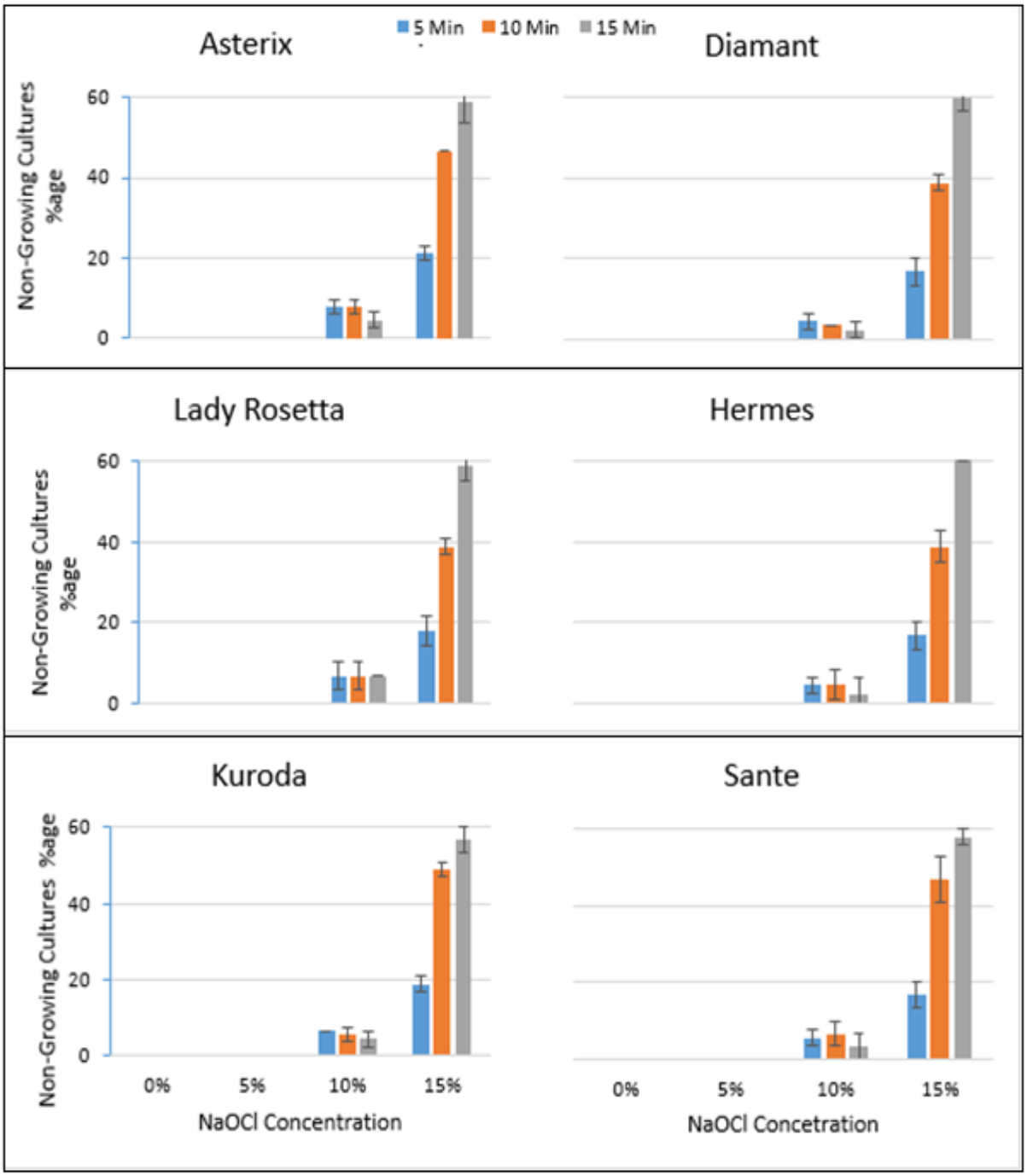

Fig. 1. Variety wise non-growing percentage of potato explants as affected by concentration and time duration of NaOCl.

Discussion

Effect of sodium hypochlorite on in vitro infection control

As expected sterilization percentage was increased significantly with the increase in concentration and dipping time of Sodium hypochlorite. This increase in the healthy plants was probably due to bleaching effects of NaOCl. Tendency of sterilization with increase in concentration and time duration was also confirmed by Ajayi *et al.* (2006).

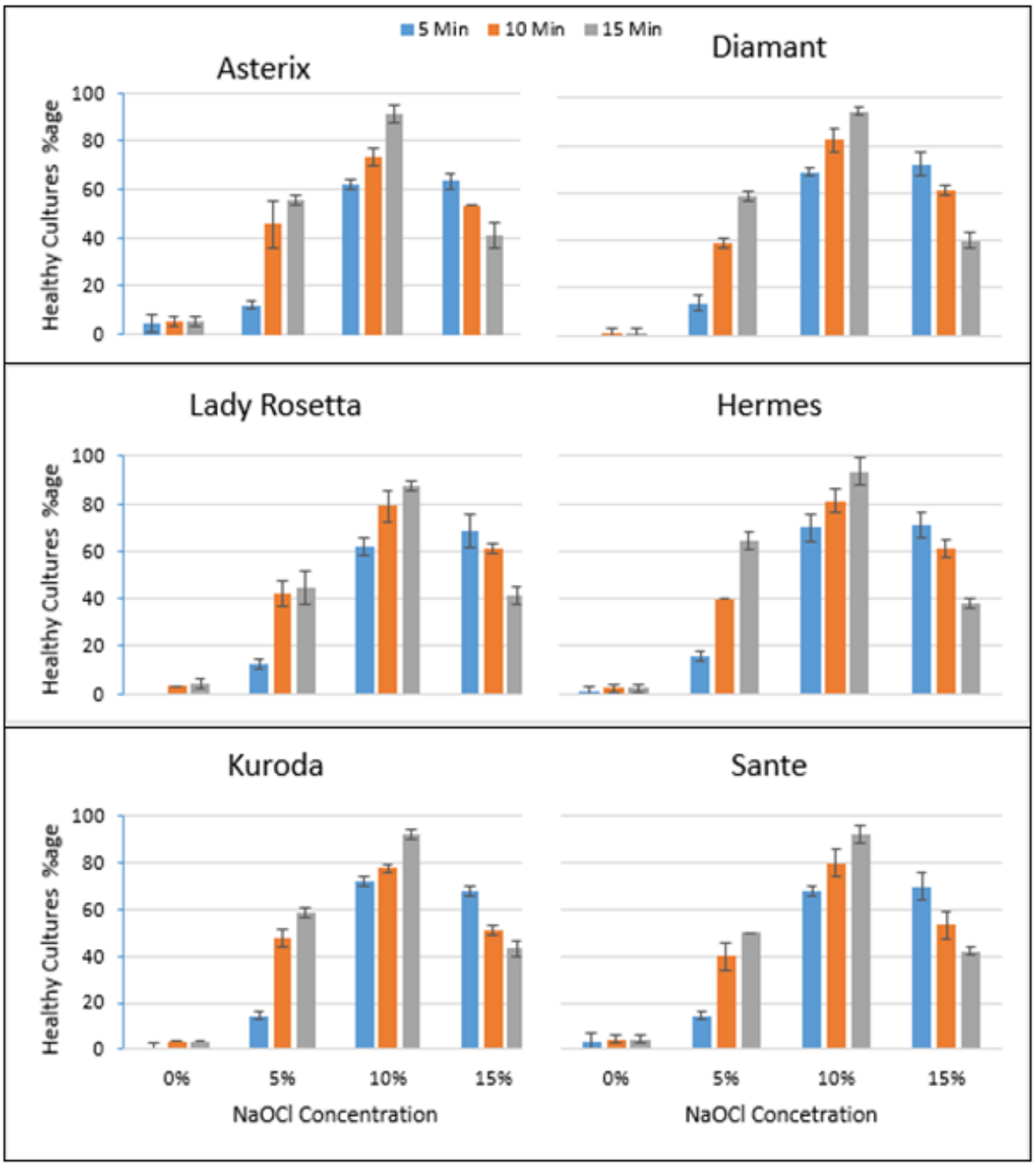

Fig. 3. Variety wise healthy cultures percentage of potato explants as affected by concentration and time duration of NaOCl.

The trend showed that concentration and time duration are inversely proportional to contamination percentage. As this move to subsequently a gradual decrease in contaminated cultures in each variety. These results are in accordance with the experiments of Badoni and

Chauhan (2010), where NaOCl was found to be better for controlling the infection of potato cv. 'Kufri Himalini'. Altaf (2006) also reported that NaOCl was effective in making clean explants of Kinow tree. These results are also in agreement with Mihaljevic *et al.* (2013). Goodwin *et al.* (1980) disinfected the sprouts of eight different cultivars with 1% aqueous Sodium hypochlorite solution. Miller and Lipschutz (1984) surface sterilized excised shoot tips in 1% sodium hypochlorite solution containing 0.1% Tween-20 for 7 minutes with gentle shaking. Naik and Chandra (1993) recommended first rinsing of sprouts with 20% ethanol for 30 seconds followed by 10 minutes shaking with 25% (v/v) sodium hypochlorite solution with 1-2 drops of Tween-20. Villafranca *et al.*, (1998) surface sterilized the sprouts with 1% sodium hypochlorite, 0.1% Tween-20 solutions for 5 minutes. There are several reports (Wescott *et al.*, 1977; Miller and Lipschutz, 1984; Naik and Chandra, 1993; Villafranca, 1998) which suggest sterilization of potato sprouts and shoot tips with 1% NaOCl for 5-10 minutes.

Effect of sodium hypochlorite on in vitro healthy cultures establishment

This decreasing trend in contamination percentage and establishment of healthy culture was probably due to bleaching effect of sodium hypochlorite by acting as an organic and fat solvent which degrade fatty acid (Estrela *et al.,* 2002). When sodium hypochlorite dissolves in water, it disintegrates into two substances i.e. hypochlorous acid and hypochlorite ion that play a role in disinfection processes (Rowe, 2013). Formation of chloramines by the action of hypochlorous (NaOCl) with fatty acid (microorganism), interfere the cellular metabolism, oxidative action with irreversible enzymatic inactivation causing lipid and fatty acid degradation (Estrela *et.al.* 2002). Sodium hypochlorite presents high surface tension (75 dynes/cm) and minimum inhibitory concentration lower than 1% for resistant microorganisms *(Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa,, Bacillus subtilis* and *Candida albicans).* The concentration rise is directly proportional to the antimicrobial effect and tissue dissolution capacity and inversely proportional to biologic compatibility, which is helpful in healthy culture establishment. Thus, considering the high surface tension of NaOCl, antimicrobial action can be achieved with the less concentrated solution and for this purpose the better option is 1% sodium hypochlorite (Bystron and Sundqvist, 1983).

Effect of sodium hypochlorite on in vitro plant mortality

Higher mortality of plants in 15% NaOCl with 10 and 15 min time duration was probably due to toxic effect of higher concentrations of sodium hypochlorite, as it also reacts with plant cell and damages the cellular membrane (Yildiz and Er, 2002). Colgecen *et al.* (2011) also found toxic effect of higher concentration of sodium hypochlorite on *Arnebiaden siflora.* Chlorine damage organic cells by oxidizing the cellular proteins as it yields free oxygen radicals. It is also reported that it disrupt the lipoprotein structure causing cytoplasm leakage and killing the cell (Roberts, 1999). These results are in accordance with Mihaljevic *et al.* (2013) and Colgecen *et al.* (2011).

Conclusion

Concentration and time duration significantly influenced the sterilization process. As both increased, percentage of healthy *in vitro* cultures increased and contaminated plants decreased. At the highest concentration (15% NaOCl) with 10 and 15 minutes dipping time there was no contaminated culture but caused mortality of explant and proved a toxic level. Whereas, the explants treated with 5% NaOCl for different time durations did not give rational number of contamination free cultures. The study has shown that the most suitable sterilization procedure for all six potato cultivars is dipping of explants in 10% NaOCl for 15 minutes.

Acknowledgement

Author Abdul Ahad Qureshi acknowledges Prof. Dr. Nadeem Akhtar Abbasi for planning and supervising the research. Author also acknowledges Higher Education Commission of Pakistan for their financial support to conduct the study and Department of Horticulture PMAS-AAUR for facilitating the research.

References

Ajayi SA, Berjak P, Kioko JI, Dullo ME, Vodouhe RS. 2006. Observation on *In vitro* Behaviour of the Zygotic Axes of Fluted Pumpkin. African Journal of Biotechnology **5(15),** 1397-1404.

Altaf N. 2006. *In vitro* bud culture of kinnow tree. Pakistan Journal of Botany **38(3),** 597-601.

Badoni A, Chauhan JS. 2010. *In Vitro* Sterilization Protocol for Micropropagation of *Solanum tuberosum* cv. 'Kufri Himalini'. Academia Arena **2(4),** 24.
http://dx.doi.org/10.7537/marsaaj020410.03

Barrett C, Casselles AC. 1994. An evaluation of antibiotics for the elimination of *Xanthomonas campestris* cv. *pelargonii* (Brown) from *Pelargonium* x *domesticum* cv. 'Grand Slam' explants *in vitro*. Plant Cell Tissue and Organ Culture **36,**169-175.
http://dx.doi.org/10.1007/BF00037716

Buckley PM, Reed BM. 1994. Antibiotic susceptibility of plant associated bacteria. Horticultural Science **29(5)**, 434.

Bystron A, Sundqvist G. 1983. Bacteriologic evaluation of the effects of 0.5% sodium hypochlorite in endodontic therapy. Oral Surgery, Oral Medicine, Oral Pathology **55(3),** 307-312.
https://doi.org/10.1016/0030-4220(83)90333-X

Chawla HS. 2003. Plant Biotechnology: Laboratory manual for plant biotechnology. Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi.

Colgecen H, Koca U, Toker G. 2011. Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebiaden siflora* L. Turkish Journal Biology **35(4),** 513–520.
http://dx.doi.org/10.3906/biy-0911-161

Constantine DR. 1986. Micropropagation in the commercial environment. In: Withers, L. and P.G. Alderson (Eds.), Plant tissue culture and its agricultural applications. Butterworth, London, p. 175-186.

Estrela CRA, Barbin EL, Spano JCE, Marchesan MA, Pecora JD. 2002. Mechanism of Action of Sodium Hypochlorite. Brazilian Dental Journal **13(2),** 113-117.
http://dx.doi.org/10.1590/S01036440200200020007

FAO. 2008. International year of the potato. Fact Sheet.
www.potato2008.org/en/potato/factsheets.html. Accessed 05 September 2016.

FAO. 2014. FAOSTAT. Statistical Database. Food and Agricultural Organization of United Nations, Rome, Italy.
http://faostat3.fao.org/download/Q/QC/E.Accessed15 February 2016.

Fernie AR, Willmitzer L. 2001. Molecular and biochemical triggers of tuber development. Plant Physiology **127(4),** 1459-1465.
https://doi.org/10.1104/pp.010764

George F. 1993. Plant propagation by tissue culture. Exergetics Ltd., Edington, England. 574.
http://dx.doi.org/10.1007/978-1-4020-5005-3

Goodwin PB, Kim YC, Adisarwanto T. 1980. Propagation of potato by shoot tip culture. Potato Research **23(1),** 9-18.
http://dx.doi.org/10.1007/BF02364021

Herman EB. 1996. Microbial contamination of plant tissue cultures. Agritech Consultants Inc, Shrub Oak, USA.

Hossain M, Ali MS, Rashid MM. 1994. Effect of inoculum levels of Potato virus Y (PVY) on yield and subsequent spread of the disease under insecticide spray and unsprayed condition. Bangladesh Journal. Botany **23,** 87.

Hussain S, Lane SD, Lane DN. 1994. A preliminary evaluation of the use of microbial culture filtrates for the control of contaminants in plant tissue culture systems. Plant Cell Tissue and Organ Culture **36,** 45-51.
http://dx.doi.org/10.1007/BF00048314

Hussey G, Stacey NJ. 1981. In vitro propagation of potato (*Solanum tuberosum* L.). Annals of Botany **48(6),** 787-796.
https://doi.org/10.1093/oxfordjournals.aob.a086186

Mihaljevic I, Dugalic K, Tomas V, Viljevac M, Pranjic A, Cmelik Z, Puskar B, Jurkovic Z. 2013. *In vitro* sterilization procedures for micropropagation of 'Oblacinska' sour cherry. Journal of Agricultural Sciences, Belgrade **58,** 117-126.
http://dx.doi.org/10.2298/JAS1302117M

Miller SA, Lipschutz L. 1984. Potato in: Ammirato P.V., Evans, D., Sharp W. R. and Y. Yasuguki (Eds.), Handbook of plant tissue culture, McMillan publishing company New York. **3,** 291-293.

Naik PS, Chandra R. 1993. Use of tissue culture technique in crop improvement with special reference to potato. CPRI, Shimla.

Odutayo OI, Oso RT, Akynyemi BO, Amusa NA. 2004. Microbial contaminants of cultured *Hibiscus cannabinus* and *Telfaria occidentalis* tissues. African Journal of Biotechnology **3(9),** 473-476.
http://dx.doi.org/10.5897/AJB2004.000-2092

Rabbani A, Askari B, Abbasi NA, Bhatti M, Quraishi A. 2001. Effect of growth regulators on *in vitro* multiplication of potato. International Journal of Agriculture & Biology **3(2),** 181-182.

Roberts K. 1999. Why does chlorine kill plant? Accessed10 March 2016.
http://www.madsci.org/posts/archives/jul99/932670591.Bt.r.html.

Rowe J. 2013. Understanding Sodium Hypochlorite. www.tpomag.com/editorial/2013/12/understanding_sodium_hypochlorite _wso.Accessed10 March 2016.

Villafranca MJ, Vermendi J, Sota V, Mingo-Castel AM. 1998. Effect of physiological age of mother tuber and number of subcultures on *in vitro* tuberization of potato (*Solanum tuberosum* L.), Plant Cell Reports **17,** 787-790.
http://dx.doi.org/10.1007/s002990050483

Wescott RJ, Henshaw GG, Roca WM. 1977. Tissue culture and storage of potato germplasm: Culture initiation and plant regeneration, Plant Science Letters **9,** 309-315.
https://doi.org/10.1016/0304-4211(77)90101-8

Yildiz M, Er C. 2002. The effect of sodium hypochlorite solutions on *in vitro* seedling growth and shoot regeneration of flax (*Linum usitatissimum*). Naturwissenschaften **89(6),** 259-261.
http://dx.doi.org/10.1007/s00114-002-0310-6

Zaman MS, Quershi A, Hassan G, Din RU, Ali S, Khabir A, Gul N. 2001. Meristem Culture of Potato (*Solanum tubersum* L.) for Production of Virus Free Plantlets. Journal of Biological Sciences **1(10),** 898-899.