Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

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OPEN ACCESS Asian Journal of Plant Sciences

ISSN 1682-3974
DOI: 10.3923/ajps.2018.173.181

Research Article
In vitro Seeds Germination and Plantlets Growth of Hot Pepper (*Capsicum frutescens* L.) On Non-autoclaved Murashige and Skoog Basal Medium

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Abstract

Background and Objective: In traditional plant tissue culture, the higher cost due to the expensive laminar air flow cabinet and autoclave. Time consuming in lengthy procedures of implementation and excessive electrical power due to unexpected power outage were the main obstacles in the success of modern plant tissue culture. By these obstacles, the use of active chlorine (NaClO) in the MS basal medium on effectiveness of contamination, seed germination and plantlet growth of hot pepper (*Capsicum frutescens* L.) *in vitro* culture was the objective of the research. **Materials and Methods:** Hot pepper seeds and MS basal medium were used as experimental materials and sterilized by 5.25% active chlorine contained in commercial household bleach in 13 various percentages of treatments ranged from 0.079-0.394% and arranged in completely randomized design (CRD). Observed variables were percentage of medium contamination, germination seed rate and percentage, plant height, leaf number, root length and root number. **Results:** The study indicated that the percentage of jar contamination during 30 days of incubation were 4 of 5 jars cultured or 80% in NaClO lower concentration (0.079%), whereas, in higher concentration (0.394%) was 1 of 5 jars cultured or 20%. Both of these concentrations had the same effect in the early of seed germination i.e., 2 days after incubation. The highest plant height was achieved in 0.394% and the highest number of leaves was noted in 0.368%, also the highest root length was in 0.105%. The root number was not of different significantly for all concentrations which range from 13.20-22.70. **Conclusion:** Hot pepper seeds germination and plantlets growth had successfully been achieved under non-autoclaved *in vitro* medium.

Key words: Active chlorine, medium contamination, hot pepper, seed germination, plantlet growth

Citation: Suaib Suaib, Norma Arief, Gusti Ray Sadimantara, Ni Wayan Sri Suliartini, Tresjia CorinaRakian and Ardhi, 2018. *In vitro* seeds germination and plantlets growth of hot pepper (*Capsicum frutescens* L.) On non-autoclaved murashige and skoog basal medium. Asian J. Plant Sci., 17: 173-181.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plant tissue culture practices in the present day become costly and time-consuming[1-7] due to the use of expensive laminar air flow cabinet and lengthy procedure and excessive electrical power resulted in higher cost and unexpected power outage in unpredictable time when sterilization is running, particularly in the regions that have suffered from these obstacles. Consequently, this procedure must be rerun so extra time will be needed to cover the processes. From these problems, alternative technique is needed to replace in which it will be more efficiently and effectively to solve the time consuming and cost effective problems in gaining good results for ranged plant tissue culture applications.

Recently, some researchers have reported the successful practices of plant tissue culture without the use the autoclave in the medium sterilization on several plant species. They were in *Musa paradisiaca* and *Ananas comosus*[1,8], *Eucalyptus pellita*[9], *Saccharum officinarum*[4,10], *Eucalyptus benthamii*[9], *Phalaenopsis*[11] and in *Chrysanthemum*[6,12]. All above reports were conducted by using conventional sterilization medium with autoclaved as control treatment. As results, those non-autoclaved sterilization media were resulted better than the conventional ones in the frequencies of contaminated media by bacterial and fungal-agents and other sources of *in vitro* medium contaminant agents.

Some agents that have successfully been reported in blocking various kinds of microfloras and microfaunas contamination medium in plant tissue cultures were plant extracts, essential oils and biosides as chemical agents. The plant extracts such as betel leaf (*Piper betle*)[13,14], turmeric (*Curcuma longa*)[15], tulsi (*Oncimum sanctum*), clove (*Eugenia caryophyllata*), datiwan (*Achyranthes bidentata*) and neem (*Azadirachta indica*)[16] had been successfully reported in eliminating the negative effects of some contaminant agents of medium in plant tissue culture.

Whilst, some essential oils have also been successfully documented in preventing the hazardous effects of several micro-organism inhibitors of *in vitro* medium in plant tissue culture, which were bergamot oil, betel oil, clove oil, cassumunar ginger oil, cinnamon oil, holy basil oil, lavender oil, lemon oil, tea tree oil and turmeric oil[4,6,17-20]. Besides, the use of biosides viz Ethanol (EtOH), Sodium hypochlorite (NaClO) and Mercury chloride ($HgCl_2$) in eradicating the contaminant agents of *in vitro* medium have also been reported elsewhere. They suggested the ability of NaClO as a sterilization agent in reducing activity of micro-organisms contaminating in *in vitro* medium[1,3-10].

The use of NaClO as an *in vitro* medium sterilant agent was widely applied in many *in vitro* research laboratories, researchers and the difference in *in vitro* techniques included various of crop plants. The advantages of this bioside was its availability as household disinfectant or as industrial bleach, affordable and easily be obtained in general markets or household shops and less poisonous when consumed by human. Also, some researchers have reported on positive effects of NaClO in cells, roots, nodes, shoots and whole plantlets development during *in vitro* culture of some plant species like pineapple (*Ananas comosus* cv Smooth Cayenne)[1], *Anthurium andreanum* Lind. cv Tropical Red[3] and two species of orchids (*Arundina bambusifolia* and *Epidendrium ibaguenses*)[20].

Hot pepper (*Capsicum frutescens* L.) was one of the staples of horticultural crop plants in Indonesia. Renowned for its determinant factor in the instability of Indonesian crop market price from year to year, Indonesian people popularly consumed higher volume of freshly hot pepper as daily diet component and for industrial human food and as human medical treatment and cosmetics industries. The burning sensation of hot pepper when contacted to the eyes or other mucus membrane was caused by the chemical content of capsaicin in the fruit. Another usage of capsaicin is as an analgesic healing of some kinds of suffering on the human skin surface[21].

By natural condition, the hot pepper cultivation was easily be implemented in the simplest way because it was germinated and grown weekly. In comparison, the *in vitro* germination of hot pepper seeds was quicker than that of the *ex vitro* germination. It takes 5-15 days for germinate in *ex vitro* culture[22-25], but only two days needed to germinate through the *in vitro* culture[26,27]. From these viewpoint, this seeds plant was therefore chosen as the object for this observation. Additionally, the study of the effect of several dosages of 5,25% NaClO contained in the household bleach (HB) and its ability to maintain the sterility of *in vitro* medium was the main objective of this study. Thus, its response to the hot pepper seeds germination and plantlets growth and development was another objective to be garnered from this study.

The significance of this study work was to determine the material preparation technique when various ways of *in vitro* procedures were applied such as micro and mass-propagation and some biotechnological practices in providing the starting materials for these techniques explained above. The *in vitro* technique utilizes some explants under free of contaminant sources as a key factor in successful

implementation of *in vitro* procedures, so that the explants used were free from the pathogenic sources or sterile. This condition must be existed and the important eligibility should be faced during *in vitro* culture. The positive effect of active NaClO in protecting *in vitro* medium from pathogenic sources and supporting the growth and development of explants during *in vitro* culture will have the broad and wide application either in the various species of plants or in the incomplete laboratory. Utilization of HB in substituting an autoclave and a Laminar Air Flow Cabinet in medium sterilization may simplify the procedure, but the various HB with the different percentage of NaClO content sold in the free market may be resulting in more frequently trials needed to determine *in vitro* procedure in some species or plant. By those significances, determination of NaClO dosage in MS basal medium as sterilant agent will provide an effective and an efficient way in gaining the pathogen free *in vitro* medium and explant/seed as well and the preparation of hot pepper seedings in micro-organisms free as a source of various biotechnological practices will succeeded in the future.

The aims of this report were to explain and to discuss the response of *in vitro* medium in contamination by pathogenic agents that were sterilized by sodium hypochlorite, hot pepper seed germination and plantlets growth and development for 30 days of *in vitro* culture. Also, the successful observation in utilization of NaClO as a medium sterilization agent and a basal medium component may be able to be applied in broader and wider aspect of plant *in vitro* culture elsewhere.

MATERIALS AND METHODS

Location and plant material: The experiment was conducted in *in vitro* laboratory Faculty of Agriculture Halu Oleo University Kendari, Southeast Sulawesi, Indonesia, during 3 months of whole project since May until July, 2017. Hot pepper seeds as material experiments were obtained from farmers in the vicinity of Kendari Municipality in which this plant had been cultivated since years ago and continuously planted until nowadays. This plant was called "cabai rawit lokal" or local variety of hot pepper (*Capsicum frutescens* L.). The Murashige and Skoog basal medium[28] was used in this experiment and fortified by 3.0 mg L^{-1} 2,4-D, kitchen sugar and solidified with agar "Walet".

MS basal medium preparations: Medium preparation was applied according to general steps practiced elsewhere except in sterilization method[29], where the procedure was carried out with slight modifications. All medium components were poured in the 1,000 mL erlenmeyer that had been filled with 600 mL sterile aquadest. The HB was then added into the erlenmeyer in the amount of desired concentration and the medium pH was measured at 5.8 or adjusted with 1.0 N NaOH or 1.0 N HCl. By using a hotplate with magnetic stirrer, the medium was homogenized and boiled for 3 min, then poured into 150 mL jars for each of 20 mL and topped with plastic transparent and sealed by rubber ring.

Seed sterilization and household bleach concentrations tested: Seeds from the pod were taken out, cleaned from debris with running tap water and the air was desiccated onto sterile petri dish layered by sterile tissue paper with 30% HB through spraying. The seed sterilization procedure were carried out by soaking them in 100 mL erlenmeyer containing ethanol 70% for 60 sec, then continued to ethanol normality with sterile aquadest in each 5 min for 3 times, then soaking them in 30% HB+two drops of Tween 30 in sterilized aquadest for 15 min. The sterilized seeds were put into another sterile petridish as the next incubation step. All seeds sterilization steps were done in outside room of laminar air flow cabinet which is in unsterilized condition.

The 13 different percentages of active NaClO in MS basal medium tested were 0.079, 0.105, 0.131, 0.157, 0.184, 0.210, 0.236, 0.263, 0.289, 0.315, 0.341, 0.368 and 0.394, respectively. These treatments were coded by the respective C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 and C13.

Experimental design and statistical analysis: *In vitro* seed germination was incubated onto culture jars according to the HB treatment tested, five seeds per jar and five jars per treatment as replications were applied so there were 65 culture jars and 325 seeds as experimental units and those treatments were arranged in a completely randomized design. The observation variables were medium contamination, seeds germination and plantlets growth and development during 30 days of incubation. All data were analyzed by one way analysis of variance (ANOVA) according to Steel and Torrie[30] through SAS statistical package for Windows version 9.13[31] and the difference between treatments was justified by Duncan's Multiple Range Test at 95% level of significance.

RESULTS

Effects of NaClO on medium contamination: From 13 different treatments of NaClO in MS basal medium, no treatment was decontaminated during 30 days of incubation. The highest percentage of active NaClO showed lower contamination frequency (20%), while the lowest one

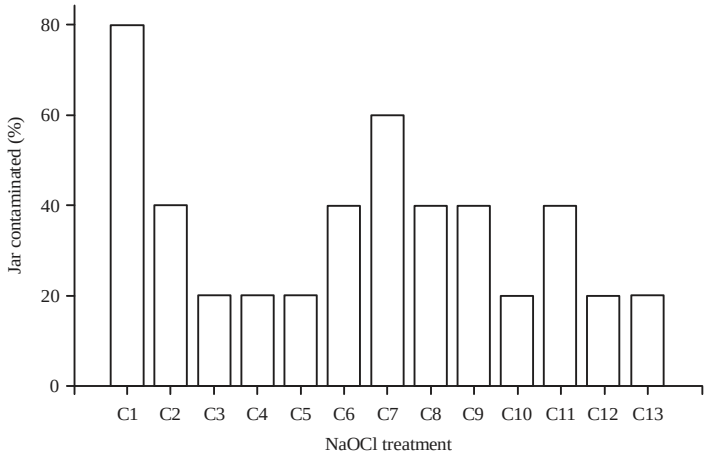

Fig. 1: Effect of 13 different percentages of active NaClO in MS basal medium on frequency of medium contamination over 30 days of incubation

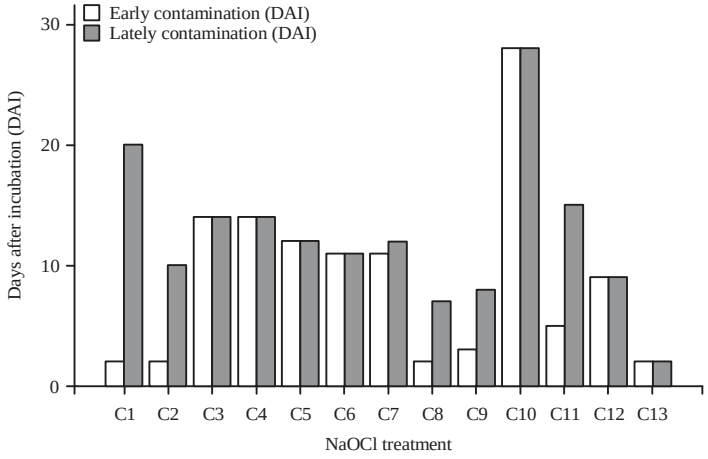

Fig. 2: Effect of 13 different percentages of active NaClO in MS basal medium on time lapse of medium free from contamination agents

indicated higher contamination frequency over 80% (Fig. 1). Meanwhile, less than 50% of treatment was contaminated during 5 days, whereas 54% of which contamination occurred after 8 days of incubation and the longest occurred when the active NaClO in the basal medium was more than 0.30% after 28 days of incubation (Fig. 2).

The effect of active NaClO in media contamination and microorganism-free time lapse media may conclude that the use 0.10% was sufficient and could be freed from contaminant sources until 28 DAI. This condition should be useful in applying the continuous induction of plant calluses as has been practiced in some biotechnological application such as callus multiplication, callus sub culture of *in vitro* somatic embryo genesis and mass of micro propagation.

Effects of NaClO on seed germination: Of the total of 325 seeds incubated, 270 of which or 83.1% was successfully germinated. A 4.1 of 5 incubation seeds were general mean value of seed germination per jar. No treatment was successfully germinate for all it seeds, but the higher rate of NaClO in MS basal medium were the respective treatments i.e. 0.131, 0.184 and 0.368%, reaching untill 96.0% of seeds were germinated. The lower rate were those remaining treatments i.e., only 64% of germinating seeds (Fig. 3).

Effects of NaClO on plantlet growth and development: Variable observed of leaf numbers was achieved in the maximum rate i.e., 6.40 leaves when MS basal medium was contained 0.315% of active NaClO (C10) and significantly

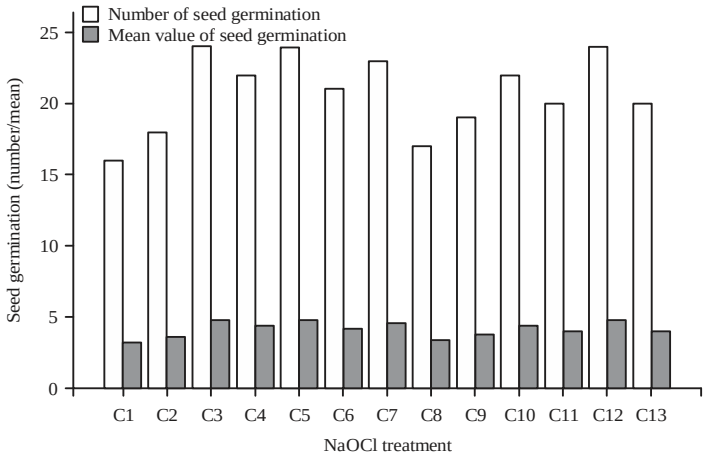

Fig. 3: Effect of 13 different percentages of active NaClO in MS basal medium on seeds germination over 30 days of incubation

Table 1: Effect of 13 different percentages of active NaClO in MS basal medium on plantlet's

NaClO concentration treatment (%)	Plantlet height (cm)	Leaf number	Root length (cm)	Root number
0.079 (C1)	5.98±0.79^{ab}	5.70±2.40^{ab}	10.08±6.56^{a}	22.70±9.19^{a}
0.105 (C2)	6.70±2.40^{ab}	4.30±0.42^{abc}	12.56±2.04^{a}	21.50±2.97^{a}
0.131 (C3)	6.33±1.34^{ab}	4.90±0.99^{abc}	8.83±0.66^{ab}	19.10±0.14^{a}
0.157 (C4)	6.25±1.51^{ab}	5.40±1.13^{abc}	7.50±1.30^{ab}	18.30±2.40^{a}
0.184 (C5)	5.80±0.03^{ab}	4.90±0.99^{abc}	8.46±2.29^{ab}	20.40±6.22^{a}
0.210 (C6)	4.33±0.61^{b}	2.40±0.28^{c}	3.97±2.62^{b}	13.20±6.22^{a}
0.236 (C7)	6.20±0.08^{ab}	3.20±1.70^{bc}	7.38±0.08^{ab}	19.90±9.19^{a}
0.263 (C8)	6.15±1.26^{ab}	5.10±2.12^{abc}	8.40±2.40^{ab}	20.80±2.83^{a}
0.289 (C9)	4.67±2.14^{ab}	4.00±0.85^{abc}	7.87±1.88^{ab}	19.90±8.06^{a}
0.315 (C10)	6.45±1.00^{ab}	6.40±1.41^{a}	9.01±0.47^{ab}	22.50±0.14^{a}
0.341 (C11)	5.10±0.17^{ab}	3.80±0.28^{abc}	10.43±0.47^{a}	16.00±4.81^{a}
0.368 (C12)	5.49±1.40^{ab}	5.30±0.42^{abc}	9.65±0.61^{ab}	22.00±0.57^{a}
0.394 (C13)	7.57±0.18^{a}	4.80±0.57^{abc}	9.09±1.03^{ab}	18.20±3.96^{a}

Means followed by the same letter in the same column are not significantly different at $p \leq 0.05$ according to Duncan's New Multiple Range Test. The results are the mean±standard error

different with 0.210% (C6), even it was not significantly different with the remaining treatments (C1-C5, C7-C9 and C11-C13; Table 1). Plant height variables indicated that the highest percentage of active NaClO treatment was produced the highest plantlet rate and significantly different with other treatments. Whereas, all the lower percentages of remaining treatments pointed out the lower rate and inconsistent values (Table 1).

The percentages effect of NaClO in MS basal medium on lengths of roots reached the highest value at the lower percentage of NaClO no more than 0.105%, but not significantly different with remaining higher percentages (Table 1). Unlike the three variables already explained above, the roots number of plantlets was not significantly different. This phenomenon indicated that the roots number was not affected by all treatments tested, but those roots formed were quite high in average. It was also expressed that the NaClO percentages were not performed the negative effect on root number of plantlets on all treatments tested (Table 1). In conclusion, plantlets performance in all treatments tested indicated the normal growth achieved especially in the plant height, leaf number and root length and number (Fig. 4).

According to the seed derived plantlets growth performances in 13 different percentages of active NaClO (Fig. 4), all of treatments indicated normally grown as seen in the complete development of roots, stem and leaves. These expressions showed the positive effect of active chlorine in growth of hot pepper seed derived plantlets, not only as medium sterilant agent but also as medium component. This latter speculation is evidently important and could be deeply observed in the future.

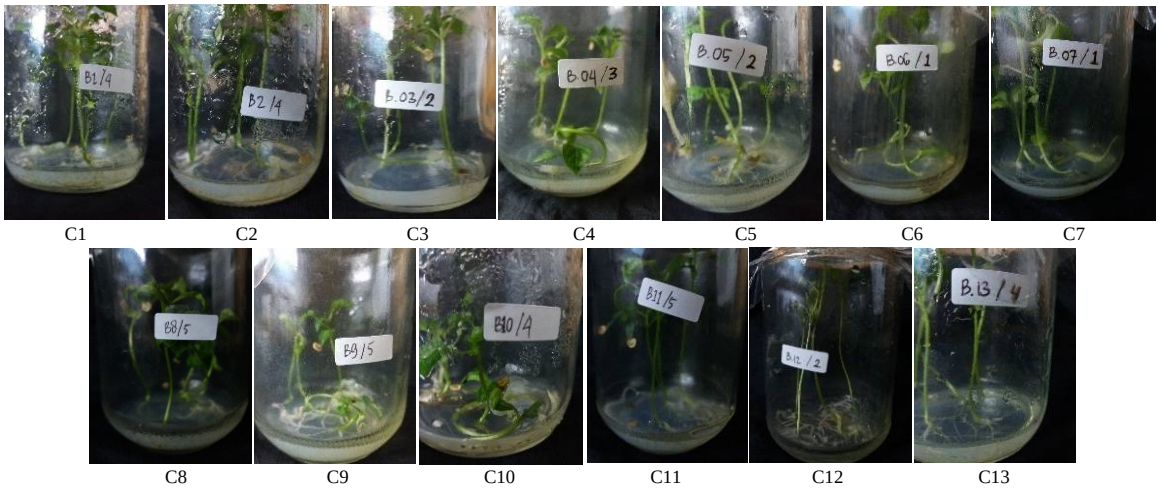

Fig. 4: Plantlets performances in 13 different percentages of active NaClO in MS basal medium over 30 days of incubation (Bars±1.0 cm)

DISCUSSION

Based on the results, the frequency of all range of treatments indicated the inconsistence patterns (Fig. 1). Not only did the lowest percentage have lower ability to keep its pathogenic free, but also was the highest one not effectively prevented yet medium contamination until 100% as indicated in all range treatments observed. Result obtained in medium contamination in this observed variable was inline with report from Rodriguez *et al.*[20] in *Arundina bambusifolia* in which 50-250 mL L^{-1} of NaClO in the MS basal medium gave the inconsistence percentage of contamination by fungal and bacterial colonies. There was no fixed pattern of the lower or higher dosage of NaClO and the minimal or maximal medium contaminated during *in vitro* incubation. In another report, Alam *et al.*[32] elucidated two NaClO percentage as treatments in media sterilization of *in vitro* micropropagation of *Cucumis sativus*. They observed the same results in media contamination.

As reported by Pais *et al.*[33] in *Gerbera hybrida* cv. Essandre, it showed that the 0.0005 and 0.001% of active NaClO in the *in vitro* media used to propagate the plant were similar to in the numbers of jar contamination i.e., five on each of them. At the lower concentration, some researcher have reported that the use of 0.01-0.05% NaClO in MS basal medium for sugarcane *in vitro* micropropagation caused 75-46% media were contaminated[4]. By using the higher concentration of sodium hypochlorite from 5-20% in micro-propagation of *Anthurium andreanum* Lind. cv. "Tropical Red" shoots did not also prevent medium contamination until 100% in which 22-28% of medium used were contaminated[3]. Conversely, other report of the 6.0% of NaClO was able to prevent medium contamination until 100% of *in vitro Chrysantemum* explant incubation[6,12]. Also, another group of researchers reported the inconsistence of frequencies of medium contamination in different concentration of NaClO into MS basal medium i.e., the 5, 10, 15 and 20% resulting in 22, 27, 22 and 28%, respectively, of culture medium were contaminated[3].

Another inconsistencies was the time lapse needed to keep the wealth of the medium. The highest percentage of active NaClO did not show longer time to prevent medium from contamination. The lowest percentage was similar to the higher one in relation to the contamination prevalence. It means that the time lapse of medium free from contaminant agents was no longer in the ranges of all these treatments. The earlier contamination of culture medium in this experiment was 2 days after incubation (DAI), but the later occurred in 28 DAI for 30 days of observation (Fig. 2).

In references of time length observation they were noted for 2 and 4 weeks after explants incubation. Similar finding has been reported in which on the 14 days after *in vitro* micropropagation of sugarcane in 0.01% NaClO of MS medium, the cultures were contaminated over 58% and the 0.2% NaClO was 56%, whilst the 0.1% NaClO was only 8% of cultures were contaminated[4]. Again, this data showed the inconsistence results of treatment ranges in which no fixed pattern was occurred relate to the treatments elevation as also reported by aforementioned author and his colleagues. This means that this data were agreed with and the results between these observations were comparable.

This also indicated that in this experiment the later expression was disagreed with this observed variable in which the departure responses of medium contamination between those reports above and this report may be due to the different in macro-climate conditions or the environmental behaviour between our laboratory and those laboratories explained, particularly in kinds and numbers of pathogenic contamination agents.

Again, these data had performed the inconsistence pattern in which it was not directly correlated with the increasing or decreasing elevation percentage of NaClO in the MS basal medium for whole treatments tested. Accordingly, the percentage ranges of NaClO from the lower (0.13%) to the higher (0.37%) had the same effect in accordance with the percentages of germinated seeds in *Capsicum frutescens* L. In another reports about seed germination in the medium which contained of NaClO of some plants indicated that the rate of germinating seed have had the different percentages. For instances, Khan and Zia[34] reported in *ex vitro* seed germination of *Limonium stocksii* have used 0-40% of NaClO in water and Zhang *et al.*[35] *in vitro* seed germination of *Paphiopedilum armeniacum* S.C. Chen et F.Y. Liu at 0-2.5% NaClO in the MS basal medium. These findings have had expressed the different percentages of germinating seed when onto the medium, either *ex vitro* or *in vitro* comprising the active NaClO as a medium component.

The different concentration or percentage of NaClO in those reports and in this observation indicated that the plantlet initiation from the *ex vitro* seeds germination has the higher percentage than *in vitro* and *in vitro* plantlet initiation from germinating seeds has the higher percentage of NaClO compared to callus induction of the explants in incubation medium. These data indicated that the existence of NaClO into the *in vitro* seeds germination medium was affected positively in germinating seeds due to its role in breaking seed dormancies, softening seed coat to facilitate the water and oxygen absorption and triggering diffusion in further seed embryo development for germination.

This important finding (Fig. 4) indicated the beneficial effect of NaClO on the hot pepper plantlet growth because the deleterious effect for all treatments tested were not occurred. The implication that the lower concentration of NaClO applied supported the good seed derived from plantlets growth and vice versa and the higher one also did not cause inhibiting growth of seed derived plantlets. The possible influence of chlorine ions by enhancing photosynthesis should be discarded, since the deficiency of this function in *in vitro* is well known[36] even though this possibility remains to be elucidated[1].

As reported by Pais *et al.*[33], the inclusion of NaClO in the organic salts of MS basal medium and White's[37] vitamins *in vitro* micropropagation of *Gerbera hybrida* cv. Essandre, the observed variables i.e., mean length of aerial part, mean root length, mean number of roots, mean number of leaves and plant dry mass were positively affected in their growth and development. The autoclaved medium as a control in their observation was not significantly different from the MS medium which was sterilized by NaClO on different percentages tested. Other reports by Rodrigues *et al.*[20] stated that the use of 50-250 mL L^{-1} of NaClO on the top of cooled MS medium by spraying method controlled the microbial contamination and promoted the explant growth and development.

Silva *et al.*[38] have reported the positive effects in seed germination and growth and development of *Byrsonima intermedia* medicinal plant through surface sterilization by NaClO in different concentrations, exposure periods and pH. These implied that the findings in this observation was similar to the prior reports, in which the NaClO component in the MS basal medium has the important role, either in eliminating microbial investation or in supporting the explant growth and development like callus and embryo induction, plantlet initiation, stem and root elongation and leaf numbers. So, the use of household bleach with 5.25% NaClO as a serialization in plant *in vitro* medium and simultaneously as a medium component could be recommended due to the positive effect in a ranged concentration according to the plant needed during the whole process of *in vitro* techniques.

CONCLUSION

Research results suggested the percentages of active chlorine in MS basal medium ranged from 0.1-0.4% can be used to sterilize nutrient medium, to germinate seeds and to grow hot pepper plantlets in *in vitro* culture. During 30 days of incubation, four of five jars medium cultured or 80% were contaminated in lower concentration (0.079% NaClO), whereas, one of five jars medium cultured or 20% were contaminated in higher concentration (0.394%). These ranges of concentrations had the same effect in the early of seed germination i.e., 2 days after incubation. The higher plant height was achieved in 0.394% and the higher number of leaves was 0.368% and the length of root was higher in 0.105%. The root number was not different significantly for all concentrations, i.e., around 13.20-22.70.

SIGNIFICANCE STATEMENT

This study reports a study on *in vitro* seeds germination and plantlets growth of hot pepper (*Capsicum frutescens* L.) on non autoclaved Murashige Skoog basal medium. Results generated here help hot pepper propagator possibly use the *in vitro* basal medium without sterilization by using expensive and long procedural scheme and high consuming electricity of autoclave. The results presented give indication the appropriate use of active chlorine in sterilization *in vitro* medium and the normal seeds germination and plantlets growth of hot pepper in using *in vitro* non-autoclaved basal medium.

ACKNOWLEDGMENT

We would like to extend our gratitude to the Dean of Faculty of Agriculture Halu Oleo University for the support provided for this research project. Also to Miss Nur Rizky Alfiany Suaib, English Language and Applied Linguistics, Faculty of Arts and Humanity, Coventry University, Priory Street, CV1 5FB, Coventry, United Kingdom, who provided proof reading for the English expressions.

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