Effects of Different Sterilization Treatments and Different Treatments of 6-Benzylaminopurine (6-BAP) on In-vitro Culture of Tomato

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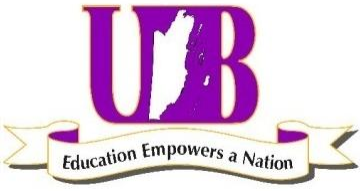

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Abstract

The study performed centralized on the effects which different concentrations of sodium hypochlorite (Clorox) have on the sterilization process of *Lycopersicon esculentum* (tomato) seeds prior to in vitro inoculation along with the effects of different concentrations of 6-Benzylaminopurine (6-BAP) on callus induction from leaves and hypocotyls segments. Tomato cultivation plays a major role in the agricultural system in the country and as such, its quality and availability are of large importance. A way of ensuring its quality and continuous production is through its micro-propagation, this includes a surface sterilization procedure and callus induction for mass production. Three concentrations of sodium hypochlorite were used, 3%, 4% and 5% to sterilize the seeds prior to inoculation and after a period of two weeks, it was observed that the seeds sterilized with a 3% sodium hypochlorite concentration had the highest yield in plant growth. After these two weeks, the plantlets produced were utilized for callus induction; from these, the leaves and hypocotyls segments were used along with three different concentrations of 6-BAP (0.2, 0.3 and 0.4 m/l). After a three week period, it was observed that the leaves set in medium containing 6-BAP at a concentration of 0.3 yielded the highest amount of callus, in the same way, the hypocotyls set in this same 6-BAP concentration showed the most callus formation as well. Overall, the leaves showed a higher yield of callus formation over the hypocotyls segments at the 6-BAP concentrations of 0.3m/l. This information can be used for further studies in search of the optimum conditions required for the best tomato plant micro-propagation.

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Table of Contents

List of Figures

List of Tables

Introduction

Tomato belongs to the Solanaceae family and it is one of the most important crops grown throughout the world [1]. It is rich in Vitamin A and C as well as in fiber [2] and it grows in tropical, sub-tropical and temperate areas [3], particularly in Peru, Ecuador, Bolivia and Andes [4]. It is produced in various countries; however, its yield is hampered by various biotic factors such as diseases, viruses, bacteria, fungi, pests and also some abiotic factors such as salinity, heat, drought and nutrient deficiencies [5]. Therefore, due to their vulnerabilities, there is a need to improve the cultivar with the use of biotechnological approaches. It is known that tomato is a favorable crop for genetic improvement and many genes for different traits have been introduced into this crop [6], but before any of these processes can be achieved, there is a need to develop a high frequency regeneration system of this cultivar in order to further advance in its genetic transformation and modification in order to yield better plants. One of the first steps that are taken is that of seed inoculation, but in order to obtain plants grown under sterile conditions free from pests, aseptic measures must be taken. The use of sodium hypochlorite has already been proven to be essential in tomato tissue culture and in vitro seed germination [7], however, it is important to find out the optimum concentration at which it needs to be used in order to increase germination yield, but at the same time obtaining no contamination. From the plants produced, it is also important to investigate the factors that affect its de-differentiation, which can include the genotype of the plant, the culture medium and the physiological stage of the explants [8]. Many researchers have already reported callus formation from hypocotyls [9] and leaves [10]. Also, a correlation between callus formation and increase in Benzylaminopurine (BAP) was studied previously [11]. Therefore, this study seeks to find the best sodium hypochlorite concentration which can be used for seed surface sterilization which would yield the highest germination rate and maintain sterilized conditions as well as the effect of different concentrations of 6-BAP on callus formation from leaves and hypocotyls segments. It also seeks to investigate if the usage of leafs, versus the usage of hypocotyls segments makes a difference in order to find out which is best explants source to be used for callus formation.

Literature Review

One of the four major world problems that modern society faces is that of food scarcity. Since the population increases day by day and medical advances aid in lengthening individual's lives, there are more people who demand food for their survival. One of the most important techniques being used now-a-days in order to address this situation is that of tissue culture, for it yields rapid and pathogen free plants [12]. Not only does it yield more, but it is recognized as a useful instrument for crop improvement. In vitro culture is used in tomato in different biotechnology applications such as the production of virus free plants [13] and genetic transformations [14]. Tissue culture can therefore be used to increase productivity of a crop through rapid availability and by cultivating superior planting material which will help assist the food scarcity issue [15].

Tomato is considered to be one of the most important crop vegetables as it not only has nutritional value and is used in a large variety of meals, but its juice can also decrease the risk of conditions such as cancer, osteoporosis and cardiovascular diseases, making them an even more important crop [16]. Therefore, tomato is considered as one of the major vegetable crops for genetic engineering, as well, it serves as a model plant for introduction to agronomical important genes into dicotyledonous crop plants [17]. In addition, its biological features, such as a relatively small genome (950 Mb) and transformability have made tomato serve as a model system for plant genetics, development, pathology, and physiology studies which has in turn resulted in the accumulation of substantial information in regards to its biology, therefore it can also be used to find more about other plants as well [18].

It is argued that genetically modifying crops is not the ideal method, however, taking into account that naturally growing tomatoes are affected by various stresses which then lead to disease and death of plant or small fruit yields, it is clear that changes need to be made and that there is a need to improve the cultivar using biotechnological approaches [6]. Since some genetic improvement work has been made in tomatoes, researchers came across a need to develop a high frequency regeneration system in the cultivar in order to further advance in the plants transformation through gene modification [6]. The first step to take before genetically transforming a plant is that of in vitro plant regeneration, and as such, the challenges facing in vitro plant regeneration, need to be addressed. In tomatoes it has been found that its regeneration

is highly dependent on the composition of the basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers [19[. In addition, since tomatoes are generally propagated by seeds, the in vitro protocols for its regeneration have not been well developed, and if the aforementioned factors affecting its regeneration are not dealt with accordingly, it can result in a large monetary loss [20]. Therefore, it is important to study the factors affecting tomatoes in vitro plant regeneration in order to develop reproducible protocols which aid in conducting the process at the optimum levels of all aspects in order to yield a higher quantity and better quality of in vitro tomato plants so that they can be used for further studies and genetic investigations, or so that they can serve as food producers.

Materials & Methods

The research was done in the University of Belize's laboratory and the better boy F1 tomato seeds were obtained from the Spanish Lookout Sunrise Market.

A) MS media

MS medium without sugar or hormones was prepared and used for invitro germination of the tomato seeds. A total of half a liter of medium was made and a ratio of 20mL/L of MS salts was used, hence, 10mL of each was added to a flask that contained a small amount of water and then filled up to the mark. The solution was warmed and agar was then added at a 7g/L ratio, hence, 3.5g were added and heated until the solution was clear and boiling. The solution was poured in a total of 17 mason jars, of which 16 contained approximately 30mL and one 20mL. These jars along with jars containing water, empty bottles, and tiles and holding utensils, which had been wrapped in aluminum foil were autoclaved.

B) Seed surface Sterilization and inoculation of seeds (better boy F1):

Seed surface sterilization and inoculation was carried out under aseptic conditions by usage of a laminar flow chamber. The seeds were sterilized in three different sodium hypochlorite solutions in order to investigate its effects on germination. The solutions prepared were set at 3%, 4% and 5% sodium hypochlorite, hence different amounts of the 5.25% sodium hypochlorite were added

to sterilized water in order to make the three 100mL solutions to be used. The first jar contained 57mL of the 5.25% sodium hypochlorite and 43mL of sterilized water in order to bring the concentration percentage to 3, the second contained 76mL of the 5.25% sodium hypochlorite and 24mL of sterilized water in order to make it a 4% concentration and the last one contained 95mL of the 5.25% sodium hypochlorite and 5mL of sterilized water in order to bring the concentration down to 5%. An approximately equal amount of tween was added to each container with the sodium hypochlorite solutions and 12 seeds were placed into each of these jars and the solution were kept in constant motion for about 5 minutes. The water was then removed and clean autoclaved water was added to all three (meanwhile kept in constant motion) in order to rinse them, this procedure was done 4 times for the 3 sets of seeds until all the soap was washed off them. Prior to inoculation, the mouth of the jars with the medium were flamed as well as the forceps being used per inoculation procedure. After placing the seeds in the medium, the mouth of the jars was again flamed before closing. All jars were labeled accordingly in order to tell them apart per sodium hypochlorite concentration. The jars were then placed in an incubation room for approximately 12 days after which germination results were obtained.

C) Callus inducing media:

A total of 600mL of callus inducing media was prepared with a ratio of 20mL/L of MS salts, hence adding 12mL of each one. It was supplemented with 3% sucrose at a ratio of 30g/L, hence 18g were added, 60g of Myo-Inositol at a concentration of 100mg/L, NAA 0.2M was added at a ratio of 50mg/100ml, hence 0.24mL was added. After these components were mixed, the solution was divided into three flasks, each containing approximately 200mL of solution, Agar was added at a ratio of 8g/L, hence 1.6g were added in every flask containing 200mL of solution. Since the solution was separated in 3 containers, these were all labeled as 0.2, 0.3 and 0.4 which would be the three different concentrations of 6-BAP being used in the research. In the first flask, a 0.2 concentration was achieved by adding 0.8mL of 6-BAP into the flask, the 0.3 concentration was achieved by adding 1.2mL of 6-BAP and the 0.4 concentration was achieved by adding 1.6mL of 6-BAP to the flask. These three flasks were then placed on a hot plate with a magnet pill inside so that the contents were well stirred as they were being heated up, once ready, the solution from the three flasks was poured into labeled mason gars at approximately

33mL per jar yielding a total of 18 jars. These were then autoclaved along with tiles, forceps and scalpels which had been wrapped in aluminum foil.

D) Inoculation of tomato leaves and hypocotyls segments on callus inducing media

The laminar flow chamber was cleaned and started up in order to maintain aseptic conditions and all material to be used was placed on it. The jars with the different 6-BAP concentrations were separated as labeled. After flaming the forceps, the plantlets were taken out from the jars and placed on one of the tiles and the leaves were cut off and separated as each plant had two leaves each. The hypocotyls was also cut into four approximately equal pieces with a previously flamed scalpel. This process was done to two plants at a time and inoculated in the jars with the callus inducing medium. The jars containing media were flamed at the mouth before placing in the plant parts and then flamed again before closing them. The holding and cutting utensils were also flamed prior to using them on the next plant, and after every 6 jars were worked on, the tile was changed in order to avoid contamination of gel from the media amongst the different concentrations. Three of the jars for every concentration contained four leaves, with the abaxial surface in contact with the media and the other 3 jars contained four pieces of the hypocotyl on each. These jars with the plant parts were then placed in a room for incubation so that they had a constant environment so that callus formation occurred.

Results & Discussions

Two sets of results were obtained; the first was from the surface sterilization with different sodium hypochlorite percentages, and the second was from the effects of the different 6-BAP concentration on callus formation from the tomato leaves and hypocotyls segments.

Surface sterilization

Table 1 show the raw data obtained 12 days after inoculation of the seeds. The percentages represent the different sodium hypochlorite percentages present in the sterilization solution used for each of the three groups of seeds and the jar numbers are the numbers given to the individual jars containing the media in which they were inoculated. As it can be observed, all the jars containing 3% and 5% contained two seeds each while the jars at 4% had two with 3 seeds and three with 2 seeds. In the table, the letter G represents ‘growth’ and ‘germination’ of the seed, for

they germinated and grew into tall plantlets while the letter N.G represent that 'no growth' occurred, meaning the seeds remained the same as when they were placed on the media. The initials G.S represent 'germination started', which in this case means that the seed showed signs of germination, but the shoot was less than one centimeter long.

Table 1: Raw data showing the effect of sodium hypochlorite on better boy tomato seeds obtained 12 days after invitro inoculation

Jar Number	3%		4%			5%	
1	G	G	G	G.S	G.S.	G.S.	N. G.
2	G	G	N. G.	N.G	G.S.	G	G
3	G	G	G	G		N.G.	G.S
4	G	G	G	G.S.		N.G.	N.G.
5	G	N. G.	G	G		N.G.	G.S.
6	G	G. S.	-			G	G.S.

None of the seeds being worked with showed any signs of contamination and the successful germination percentage obtained, as seen in Table 2, showed that the highest germination rate was achieved by the 3% sodium hypochlorite with 83% while the lowest was obtained by the 5% sodium hypochlorite concentration with 25%. When using 4% concentration, there is a 50% chance that the seed will germinate and as it can be seen in the results, the higher the sodium hypochlorite concentration, the lower the chances for successful seed germination to occur. This can also be observed in Figure 1 as it portrays a visual summary of the results obtained.

Table 2: Effect of sodium hypochlorite on survival and germination of better boy tomato seeds as seen 12 days after invitro inoculation of the seeds

Sodium hypochlorite Concentration	No. of Seeds per Concentration	Seeds that Germinated (%)	Seeds that didn't Germinate (%)	Seeds that initiated Germination (%)	Seeds Contaminated (%)	Germination Rate (%)
3%	12	83.0	8.3	8.3	0	**83**
4%	12	50.0	16.7	33.3	0	**50**
5%	12	25.0	41.7	33.3	0	**25**

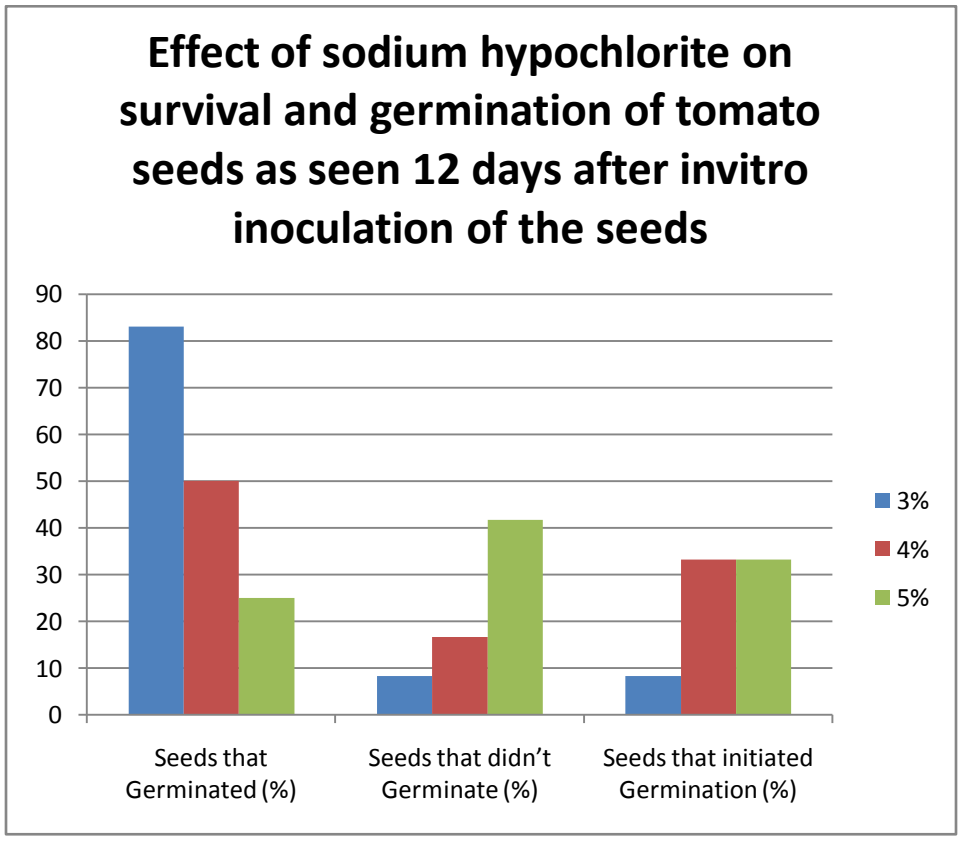

Figure 1

It was observed that 4% and 5% the percentage of seeds that showed signs if initiation of germination remained constant, while 5% had the most seeds which did not germinate. Therefore, the optimum sodium hypochlorite concentration for seed surface sterilization proved to be 3% as it showed no contamination and the highest yield of germination.

Callus formation from abaxial surface of leaves vs. hypocotyls segments

Three 6-BAP concentrations were used in order to find its effect in callus formation in tomato plants. The three concentrations used were 0.2, 0.3 and 0.4M. Three observations were made and are represented in tables 3, 4 and 5 in which C.F represents callus formation, I.C.F represents initiation of callus formation and N.C represents no callus. The observations made were done by individual leaf and hypocotyls segment in each of the different concentrations, this would allow a less generalized view on the results. Each table has the callus formation percentage which was based on the total number of plants and the total number of plants that showed callus formation (C.F).

Table 3: Data obtained from the leaf and hypocotyls inoculation in callus inducing media containing a 0.2M 6-BAP for approximately 15 days

Jar Number	Leaves		Hypocotyl Segments			
1	C.F	I.C.F	C.F	I.C.F	I.C.F	N.C
2	I.C.F	I.C.F	C.F	C.F	I.C.F	N.C
3	C.F	C.F	N.C	N.C	N.C	N.C
Callus Formation Ratio (%)	**50.0**		**25.0**			

Table 4: Data obtained from the leaf and hypocotyls inoculation in callus inducing media containing a 0.3M 6-BAP for approximately 15 days

Jar Number	Leaves		Hypocotyl Segments			
1	C.F	C.F	C.F	I.C.F	N.C	N.C
2	C.F	I.C.F	C.F	C.F	I.C.F	N.C
3	C.F	C.F	C.F	C.F	I.C.F	N.C
Callus Formation Ratio (%)	**83.3**		**41.7**			

Table 5: Data obtained from the leaf and hypocotyls inoculation in callus inducing media containing a 0.4M 6-BAP for approximately 15 days

Jar Number	Leaves		Hypocotyl Segments			
1	C.F	C.F	I.C.F	I.C.F	N.C	N.C
2	C.F	C.F	N.C	N.C	N.C	N.C
3	I.C.F	I.C.F	C.F	C.F	I.C.F	N.C
Callus Formation Ratio (%)	**66.6**		**16.7**			

The percentages of callus formation, initiation of callus formation and of no callus were calculated for both, the leaves (Table 6) and the hypocotyls (table 7). Visual representations of this information can be seen in Figure 2 and 3 in which it is seen that for callus formation in leaves, the usage of 0.3M 6-BAP was the best and for callus formation from hypocotyls, this was also the case, even if the number figures were lower than those obtained from the leaves.

Table 6: Effect of different concentrations of 6-BAP on leaves inoculated in callus inducing media as seen 15 after invitro inoculation

6-BAP Concentration (M)	Callus Formation (%)	Initiation of Callus Formation (%)	No Callus (%)
0.2	50.0	50.0	0
0.3	83.3	16.7	0
0.4	66.6	33.3	0

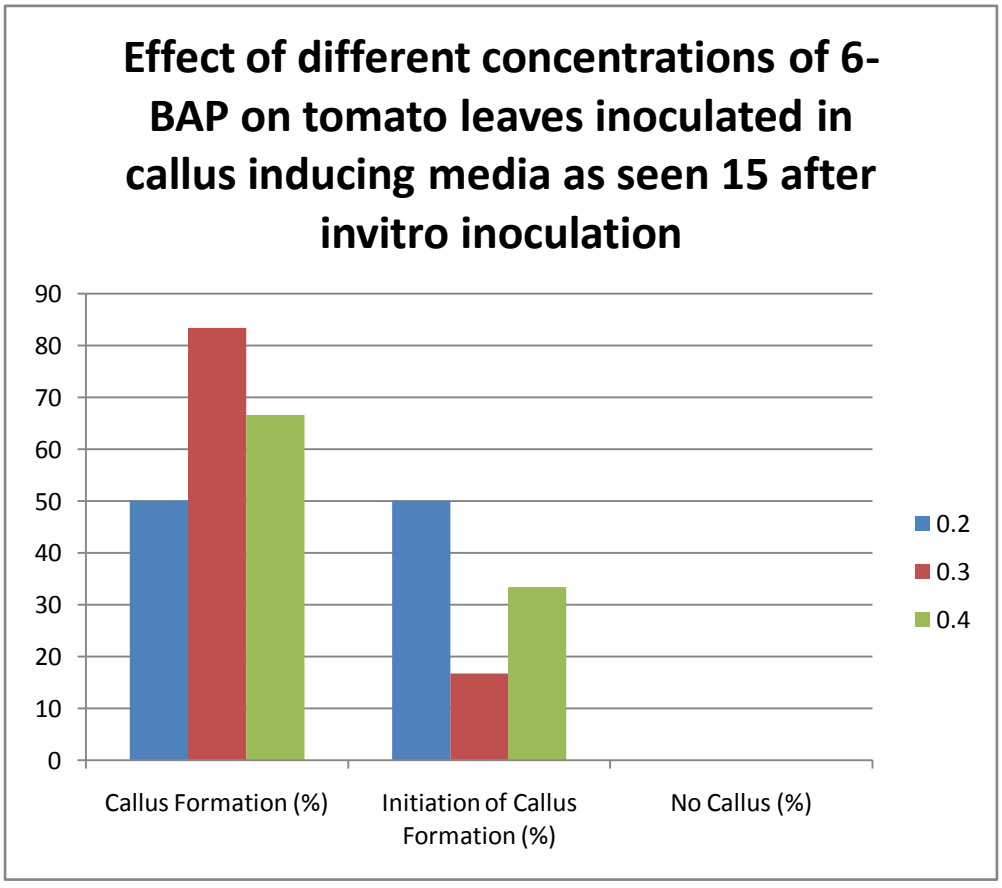

Figure 2

Table 7: Effect of different concentrations of 6-BAP on hypocotyls inoculated in callus inducing media as seen 15 after invitro inoculation

6-BAP Concentration (M)	Callus Formation (%)	Initiation of Callus Formation (%)	No Callus (%)
0.2	25.0	25.0	50.0
0.3	41.7	25.0	33.3
0.4	16.7	25.0	58.3

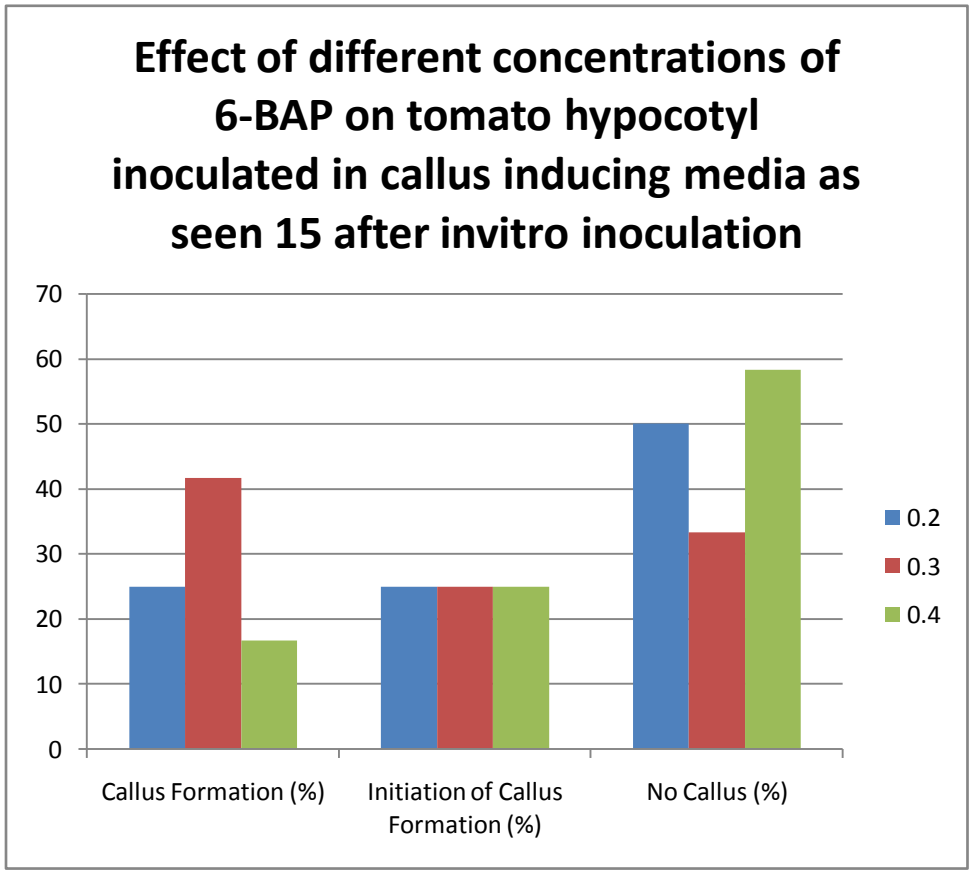

Figure 3

Conclusions

Sodium hypochlorite's concentration in the solution used for the surface sterilization proved to affect the germination process of tomato seeds. In this study it was observed that the higher the concentration, the less seeds germinated. The concentrations used were of 3%, 4% and 5% and the percentage which yielded the most germinated seeds was that of 3% followed by 4% and 5% had the minimal germination. Callus induction was observed from the leaves and hypocotyls segments at various degrees, these being callus formation, initiation of callus formation and no callus. These depended on the 6-BAP concentration being used as well as the plant segment being used. Over all, the leaves at a 0.3 concentration yielded the highest callus formation, however, when comparing the results amongst the hypocotyls, the ones at 0.3M also proved to yield the highest callus formation, even if its amount was lower than that obtained from the leaves. Therefore, in this study, using 3% sodium hypochlorite for surface sterilization of the seeds and a 0.3 concentration of 6-BAP proved to be the best for germination in aseptic conditions and callus induction respectively.

Recommendations

Throughout the process of the research, some aspects could have been improved in regards to the aseptic conditions during the inoculation of the leaves and hypocotyls segments prior to callus formation as some of the jars contained partial contamination. Another aspect that needed improvement was that of the procedure in which the callus inducing media was made, as adding the agar prior to heating the solution could have had an effect in the results obtained. It is also important to duplicate the research in order to have more significant results, as following the same procedure twice can make the data obtained more concise and credible, also, increasing the number of explants being used in order to have more treatments to compare against each other to prove that the results are significant rather than one time results. It would have also been important to make more observations for the callus induction phase in order to find out which started to form callus first and to investigate the inhibitory effects the contaminants might have caused on the callus formation.

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