South Western Journal of Horticulture, Biology and Environment
P-Issn: 2067- 9874, E-Issn: 2068-7958

Vol.11, No.2, 2020
pp.67-81
Art.no. e20106

EFFECT OF GROWTH REGULATORS ON MICROPROPAGATION AND *IN VITRO* TUBERIZATION OF *Solanum tuberosum* L. cv. vermosh

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ABSTRACT. *Potato conventional propagation is asexual by using the tubers which allows the dissemination of pathogens to new cultivation areas. Thus, biotechnological techniques based on tissue culture are very important for obtaining a healthy planting material. The aim of this study was to evaluate the effects of different combinations and concentration of growth regulators i.e., BAP, kinetin and IAA on micropropagation and in vitro tuberization of potato cv. Vermosh grown in northern Alps of Albania. For in vitro proliferation, BAP and kinetin in two levels (0.5 and 1 mg/l) were examined, while for in vitro tuberization, eight hormonal combinations kinetin/IAA and BAP/IAA ratios were tested. The proliferation of the explants into plantlets was varied depending on the treatments. Related to shootlets and root lengths, a slight efficiency of kinetin in comparison to BAP was observed. Higher concentrations of BAP or kinetin (1 mg/l) caused decrease in biometric parameters except leaves number. The higher percentage (53.3%) of tuberization was observed with the treatment which contained BAP 2 mg/l + IAA 1 mg/l. However, the lower tuberization percentage (25.9%) was obtained on kinetin 2 mg/l + IAA 0.5 mg/l containing medium. On the other hand, it is observed that, tuberization from under medium stolons was induced in a higher percentage for all treatments in comparison to the areal tuberization. The results proved that the technique used in this study can be applicable for in vitro multiplication and microtuberization of potato cv Vermosh and might be a possible for in vitro cloning of other potato cultivars.*

KEY WORDS: *potato, in vitro propagation, microtubers, phytohormones ratio.*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most cultivated crops in the world besides wheat, rice and corn (Dowling 1995) due to its importance as a source of carbohydrates, minerals and vitamins. Potato tuber has a lot of nutritional energy and protein per unit (McGill 2013) and, in this context, it is considered one of the principal food sources for people having thus a great socio-economic impact. Nowadays the world annual potato production does not fully satisfy the needs of the ever-growing population, as production is heavily influenced by temperature, humidity, soil composition, and intensity of light. In Albania, cv. Vermosh is cultivated in the northern Alps of the country, and although it has a good resistance to diseases and pests, nowadays it does not present the qualities that once existed (Jani & Elezi 2017). There is no uniformity in plant and tuber growth and does not produce the desired output. Over the years, it has not been worked too much for seed selection and production, which has led to the collapse of this cultivar and nowadays, it is at risk of extinction (Jani & Elezi 2017). On a commercial scale, potato has vegetative propagation by tubers but in this type of multiplication contamination rates can be very high thus decreasing plant productivity and causing vigor losses (Nikitin et al. 2018). So, it is extremely important finding alternative propagation methods that provide growth in production in a short time and throughout the year. The controlled and aseptic environment of tissue culture laboratory provides optimal conditions for multiplication of healthy plant materials.

There has been a worldwide tendency in the use of *in vitro* techniques for the micropropagation and virus-free potato plants production. For this purpose, meristem culture and *in vitro* tuberization is extensively used (Dodds 1988). Due to their small size and weight, microtubers can be stored easily and also have advantages related to transportation and production practices. They can be planted directly on land and can be produced in quantities of whatever season. Microtubers have similar morphological, biochemical and genetic characteristics compared to tubers produced in the field and consequently, it is considered as very important optimizing methodologies for mass production of potato microtubers, a process which will have a great impact in world potato production (Kanwal et al. 2006). In this respect, it is frequently reported that the use of different types and/or combinations of growth regulators highly affect *in vitro* induction of potato microtubers (Tugrul et al. 2001, Coleman et al. 2001).

Despite this, other physical and chemical factors, such as photoperiod and other nutrient components, and potato genotype also are reported as important factors that trigger *in vitro* tuberization in potato (El Sawy et al. 2007, Aksenova et al. 2009, Aslam & Iqbal 2010, Nistor et al. 2010). Besides conventional methods, potato propagation and microtubers production has been improved by using innovative techniques such as bioreactors thus significantly increasing growth rates and enhancing tuberization process (Rahman et al. 2015). The purpose of this study was to compare two different cytokinin types and various PGRs ratios with the aim of stabilization of a micropropagation and *in vitro* tuberization protocol of potato cv Vermosh using apical sprout explants.

MATERIAL and METHODS

Plant material, disinfection and sterilization

Plant materials source: The potato tubers were collected from Vermosh region, Northern Alps in Albania. The experiment was carried out at the Plant Cell and Tissue Culture Laboratory, Department of Biotechnology, Faculty of Natural Sciences, University of Tirana.

Tuber sprouting and explant sterilization: The potato tubers were well washed with running tab water and soaked in GA_3 solutions at 30 ppm for 60 min. to induce their rapid sprouting. As initial explants were used 10 days old buds (after tuber sprouting) (Fig. 1-a). The isolated apical buds were disinfected with 70% ethanol for 3 minutes and were sterilised with 0.01% $HgCl_2$ for 5 minutes (Fig. 1.b). Finally, the explants were rinsed three times with sterile distilled water.

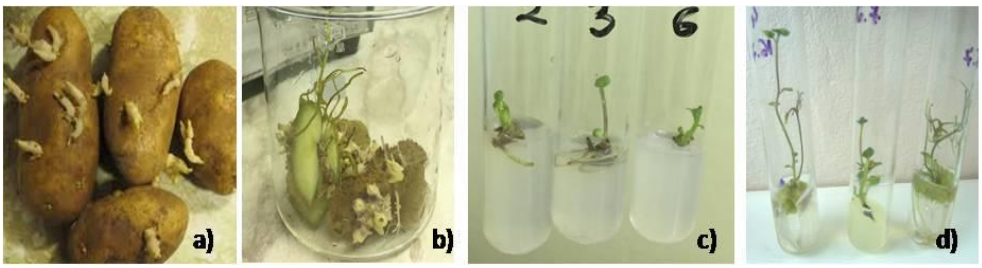

Figure 1. a) Potato tuber sprouting b), isolation and sterilization of explants, c) proliferation of shootlets and rhizogenesis after 2 weeks of sprouts culturng d) and caulogenesis and rhizogenesis after 4 weeks of culturing.

Table 1. Media composition for *in vitro* proliferation and tuberization stages.

Inoculation and proliferation stage			
Treatments	Kinetin (mg/l)	BAP (mg/l)	IAA (mg/l)
I	0.5	-	-
II	1	-	-
III	-	0.5	-
IV	-	1	-
Tuberization stage			
I	1	-	0.5
II	1	-	1
III	2	-	0.5
IV	2	-	1
V	-	1	0.5
VI	-	1	1
VII	-	2	0.5
VIII	-	2	1

Media composition for proliferation and tuberization

Proliferation experiment: Initial explants were cultivated on MS media (Murashige & Skoog 1962) and the effect of benzylaminopurine (BAP) and kinetin was evaluated (in concentrations of 0.5 and 1 mg/l each) (Table 1). In all cases, the media was enriched with 3% sucrose and 0.6% agar. The pH value was stabilized at 5.7 prior to autoclaving at 121ºC or psi for 20 min. (Kongjika et al. 2012).

Microtuberization experiment: About a month after the beginning of the *in vitro* culture, the proliferated plantlets were transferred to tuber induction media. In order to test and to stabilize the *in vitro* tuberization protocols, eight hormonal combinations of kinetin/IAA and BAP/ IAA ratios were tested (Table 1).

Growth chamber conditions

After labelling the date of culture, and type of the experiment and/or treatment, all cultures are maintained in the growth chamber with the controlled physical parameters (25° ± 2°C in a 16h/8h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m^{-2} s^{-1}).

Data elaboration

For all treatments, during the proliferation stage, roots number/explants, roots length/explants, shoots number/explants and leave number/explant were evaluated. All biometric data are presented as mean ± standard deviation. During tuberization stage the positive or negative response for surface or in medium

tuberization, rhizogenesis and callogenesis for 8 treatments were evaluated. A binary logistic regression model was applied to all the tuberization media treatments to find if type of cytokinin and/or its concentration had a significant effect on the percent of tuberization from superficial stolons, tuberization from in medium stolons, overall tuberization, rhizogenesis and callogenesis. Success of tuberization was coded as binary variable for each explant (1 when for positive response and 0 for lack of tuberization induction). Correlation analysis to understand relations between different developmental processes was performed with Pearson correlation coefficients. All data was processed in the IBM SPSS Statistics 20 software.

RESULTS

Growth parameters during proliferation stage

In the present experiment, callus formation shoot proliferation and ability of rhizogenesis were investigated. It was found that, explants respond positively to all treatments during proliferation stage. In most of the explants, the development of a significantly differentiated callus mass, the regeneration of multiple shootlets and rhizogenesis were observed in all treatments. Callogenesis induction was observed in the first days of culture. The shoots were differentiated directly by meristem sites of initial explants (Fig. 1-c) or indirectly from callus cultures callus (Fig 1-d). After two weeks of culture, biometric parameters as root number, root length, leaf number and shoots length for four hormonal treatments were measured. From the results, it was noted that in most cases there are not observed significant differences between BAP and kinetin at the same concentration (0.5 mg/l). Except for leaves number parameter, at higher concentrations of BAP and kinetin (1 mg/l), a decrease in biometric parameters rates was observed (Fig. 2). Related to shootlets and root lengths, a slight efficiency of kinetin in comparison to BAP was observed.

In vitro tuberization

For the purpose of obtaining a great number of plantlets, the shoots obtained from the stage I underwent a subculture stage where the nutrient media was the same as in the first stage for each treatment. Thereafter, the plantlets were inoculated in 8 hormonal treatments (Table 1) for the induction of *in vitro* tuberization process. They were kept in such conditions

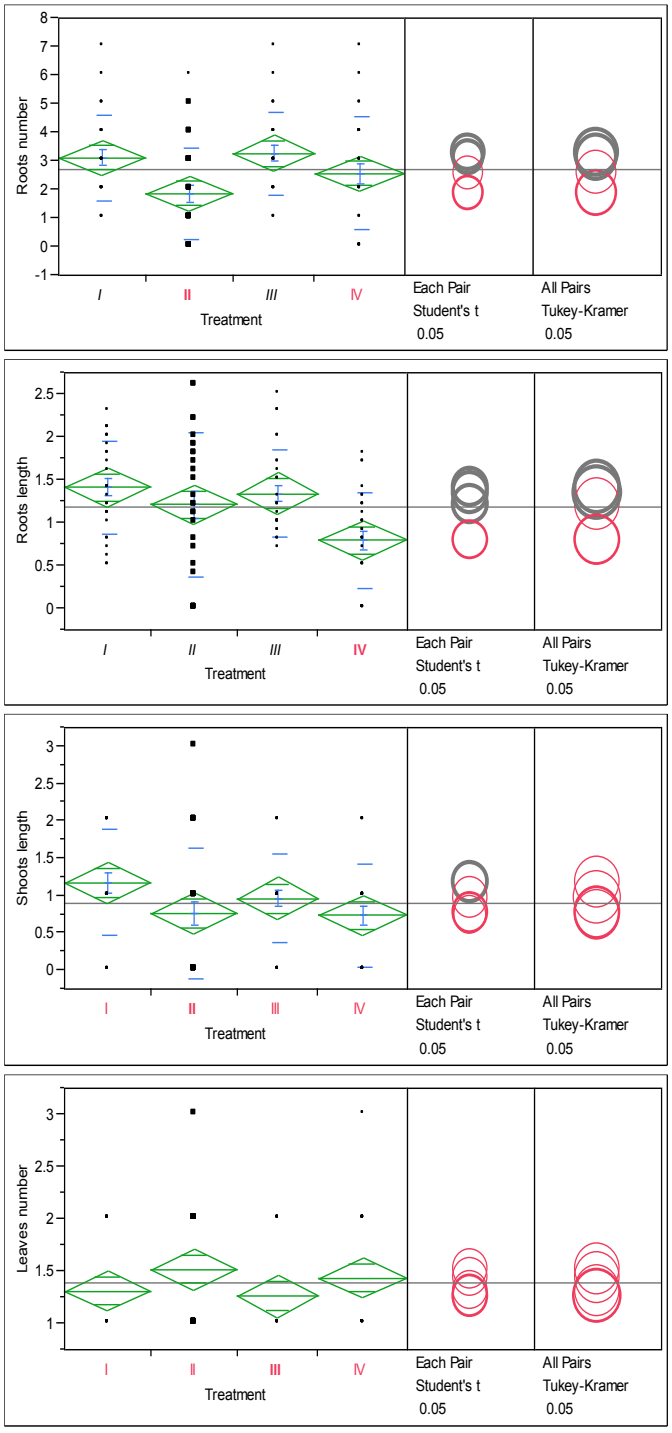

Figure 2. Evaluation of biometric parameters during proliferation stage in the four growth regulators treatments after 2 weeks of culturing.

for a period of 30 days without passing into subculture in order to induce microtubers. It was observed that the plantlets present different development processes depending on the growth regulators combinations. In many of them there was observed *in vitro* tuber formation and also the formation of callus and/or the formation of a root system. Depending on the hormonal treatment different rates for each process are observed. *In vitro* tuberization process was observed in all hormonal treatments, beginning with the formation of stolons. Node explants show two types of tuberization processes: tuber formation from underground stolons (stolons grown under the nutrient media and emerging from the base of the shoots) (Fig. 3) and tuber formation from surface (areal) stolons (Fig. 4). It was noticed that the major part of explants shows only one type of tuberization. In these conditions the tuberization induction rate separately for each tuberization type and total tuberization rate was evaluated (Fig. 5). It is observed that, tuberization from underground stolons was induced in a higher percentage for all treatments in comparison to the other tuberization type (Fig. 5). The higher rate for this type of tuberization, 53.3%, was observed in VII treatment which contained BAP 2 mg/l and NAA 1 mg/l. Meanwhile the lower tuberization rate was observed in the II and III treatments, respectively 27.6% and 25.9%, containing the former kinetin 1 mg/l and IAA 1 mg/l and the later kinetin 2 mg/l and IAA 0.5 mg/l. Besides all these differences in the percentage of tuberization, the logistic regression model for tuberization induction from surface stolons is not statistically significant, $\chi^2(7) = 7.246$, $p < 0.05$. The model explained 4.3% (Nagelkerke R^2) of the variance for this process and correctly classified an overall percentage 61.7% of cases (Table 2). Regarding tuberization induction from surface stolons, the best result (28.6%) was observed in VI nutrient media containing BAP 1 mg/l and IAA 1 mg/l, meanwhile the lower percentage rate (7.15%) is observed in IV nutrient media containing kinetin 2 mg/l and IAA 1 mg/l (Fig. 5). Even in this case, the logistic regression model is not statistically significant $\chi^2(7) = 7.255$, $p < 0.05$. The model explained 4.8% (Nagelkerke R^2) of the variance for this process and correctly classified with an overall percentage 78.1% of cases (Table 2). It's worth to mention that, the lower percentages for both types of tuberization were observed in kinetin containing media. For rhizogenezis induction, the best results were observed in the II nutrient media containing 1 mg/l kinetin and 1 mg/l IAA, respectively 65.5%. The VI nutrient media containing 1 mg/l BAP and 1 mg/l IAA gives similar results, respectively 64.3% (Fig.6). In general, there

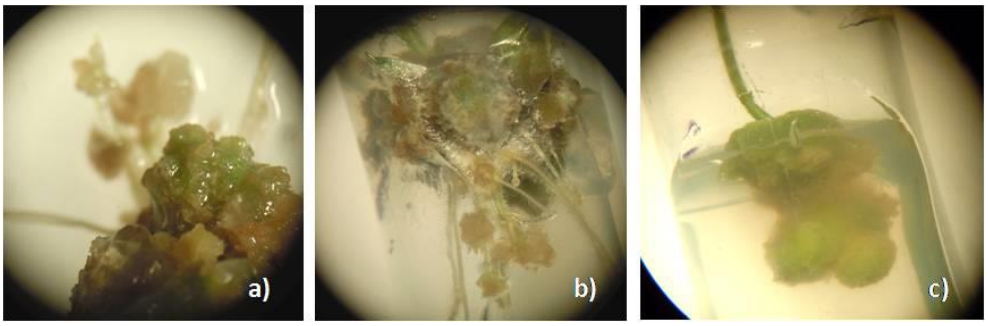

Figure 3. *In vitro* tuber formation from underground stolons a) initial of microtuberization b) microtubers formation c) minitubers formation.

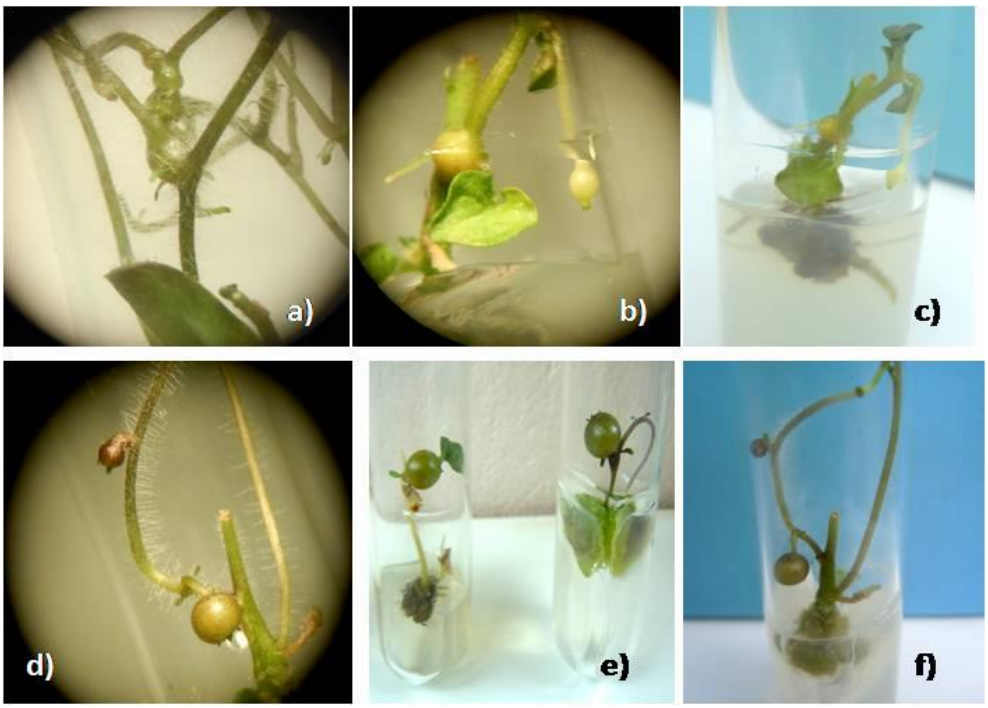

Figure 4. *In vitro* microtuber formation from areal stolons: a) initial of microtuberization, b, c) stolons and microtubers formation and d, e, f) minitubers formation.

were no significant differences between BAP and kinetin containing media. A logistic regression is performed to ascertain the effect of hormonal treatment on the rhizogenesis induction process (Table 2). The logistic

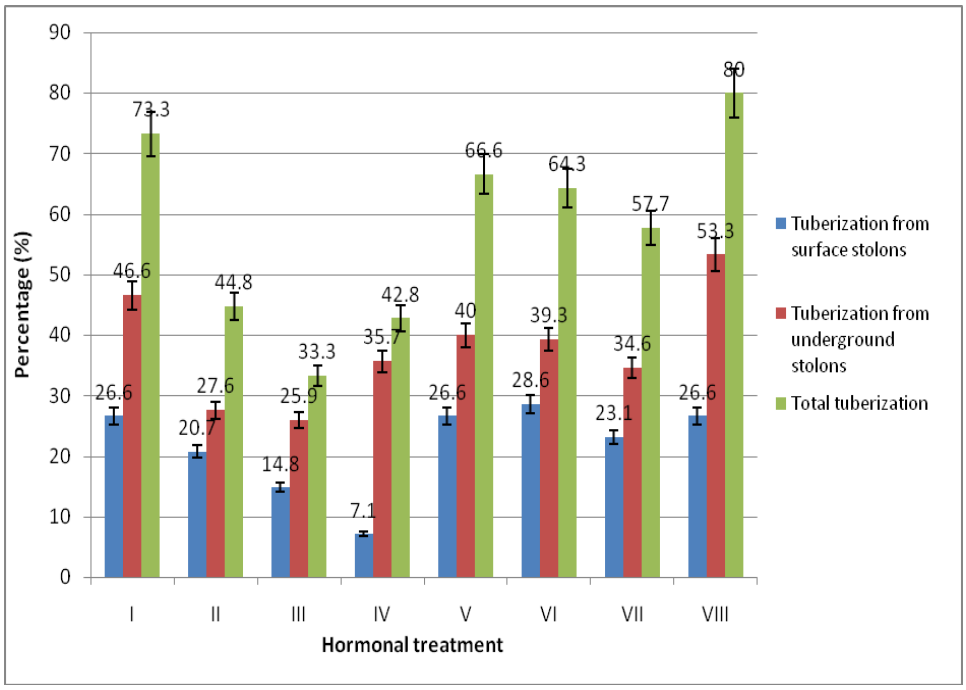

Figure 5. Tuberization rate according to hormonal treatments

Table 2. Omnibus tests of model coefficients and model summary.

	Chi-square	df	Sig.	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
Tuberization from underground stolons	7.246	7	0.404	295.917[a1]	0.031	0.043
Tuberization from superficial stolons	7.255	7	0.403	232.610[a3]	0.031	0.048
Rhizogenesis	10.801	7	0.148	305.275[a2]	0.046	0.062
Callogenesis	15.116	7	0.035	267.704 [a1]	0.064	0.090

a1; a2; a3. Estimation terminated at iteration number specifically 4, 3 and 5 because parameter estimates changed by less than .001.

regression model is not statistically significant, $\chi^2(7) = 10.801$, $p < 0.05$. The model explained 6.2% (Nagelkerke R^2) of the variance in rhizogenesis induction and correctly classified an overall percentage 58.3% of cases. In concern of callogenesis induction, the rates are relatively high in all

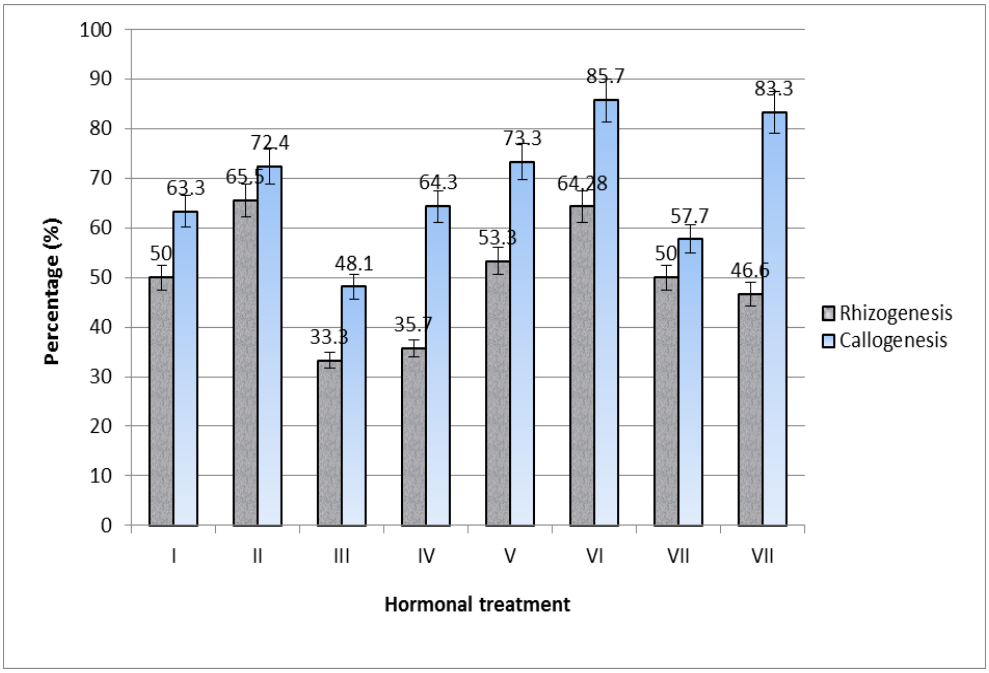

Figure 6. Callogenesis and rhizogenesis rates according to hormonal treatments.

hormonal treatments (Fig. 6). The VI and the VIII nutrient media, specifically the former containing 1 mg/l BAP and 1 mg/l IAA and the latter containing 2 mg/l BAP and 0.5 mg/l IAA give the best results, respectively 85.7% and 83.3%. In this case, logistic regression model is statistically significant $\chi^2(7) = 15.116$, $p < 0.05 = 0.035$ (Table 2). The model explained 9.0% (Nagelkerke R^2) of the variance between treatments in callogenesis induction and correctly classified an overall percentage 69.3% of cases.

Pearson correlation coefficients were determined to study the strength of relation between different developmental processes observed under these conditions (Table 3). A significant positive correlation can be seen between rhizogenesis and callogenesis (r = 0.540) rhizogenesis and overall tuberization process (r = 0.191), meanwhile, a significant negative correlation can be seen between rhizogenesis and tuberization from surface stolons (r = -0.191). Furthermore, a remarkable positive correlation was also determined between callogenesis and tuberization from underground stolons (r = 0.509) meanwhile a negative correlation is observed between callogenesis and tuberization from surface stolons (r = -0.191). Otherwise, tuberization from surface stolons is negatively correlated

Table 3. Pearson correlation between the observed developmental processes.

Specification		Rhizogenesis.	Callogenesis.	Tuberiz. from surface stolons	Tuberiz. from underground stolons	Total tuberization
	N	228	228	228	228	228
Rhizogenesis	Pearson Correlation	1	0.540**	-0.191**	0.298	0.151**
	Covariance	0.251	0.126	-0.040	0.073	0.037
Callogenesis	Pearson Correlation	0.540**	1	-0.079	0.509**	0.411
	Covariance	0.126	0.215	-0.015	0.115	0.094
Tuberiz. from surface stolons	Pearson Correlation	-0.191**	-0.079	1	-0.351**	0.426
	Covariance	-0.040	-0.015	0.172	-0.071	0.087
Tuberiz. from underground stolons	Pearson Correlation	0.298**	0.509**	-0.351**	1**	0.664**
	Covariance	0.073	0.115	-0.071	0.237	0.160
Total tuberization	Pearson Correlation	0.151*	0.411**	0.426**	0.664*	1**
	Covariance	0.037	0.094	0.087	0.160	0.244

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed)

with tuberization from underground stolons (r = -0.351), whereas a strongly positive correlation is observed between the later process and rhizogenesis (r = 0.298), callogenesis (r = 0.509) and overall tuberization process (r = 0.664). Putting it all together, it results that the overall tuberization process is positively correlated with all the other developmental processes observed under these conditions.

DISCUSSIONS

The plant growth regulators are important factors that affect callus induction; shoot proliferation and root formation in plant tissue culture studies (Cutler & Boneta 2009). Therefore, we investigated the effect of kinetin or BAP in two concentrations (0.5 and 1 mg/l) on regeneration of potato cv. Vermosh. The results obtained indicated that callus cultures were induced and direct and indirect regeneration were obtained on the two levels of BAP or Kinetin. Obviously 0.5 mg/l concentration of the both cytokinins was more effective comparing with 1.0 mg/l. Moreover, kinetin had a slight efficiency on shootlet and root lengths in comparison to BAP at the same level. These results are supported by the findings of Nagib et al. (2003) who stated that the combination of 0.5 mg/l GA_3+ 0.04 mg/l Kin was effective for stabilizing the first stage of meristem culture for various potatoes varieties. However, Badoni & Chauan (2010) found as highly effective the use of kinetin 0.01 mg/l combined with NAA 0.1 mg/l for *in vitro* development of meristem tip and multiplication of potato plantlets. On the other hand, Koleva et al. (2012) mentioned that the addition of 2 mg/l BAP into proliferation medium of potato gave higher number of shoots/explant. Similarly, in their study on the effects of different concentrations of 6-benzylaminopurine on proliferation of potato grown *in vitro*, Rabbani et al. (2001) found that 2 mg/l BAP was the best level for number shoot proliferation. Recently, Emaraa et al. (2017) determined the optimal types and concentrations of plant growth regulators as well as sucrose concentrations on micropropagation and microtuber formation in potato and found that the highest multiplication aspects were obtained by MS medium containing 0.2 mg/l NAA together with 0.2 mg/l Kin. Regarding *in vitro* tuberization of potato several authors attribute an important role in this process to the use of cytokinins such as BAP (Zhang et al. 2005, Sarkar et al. 2006) and kinetin (Coleman et al. 2001, Aksenova et al. 2009).

Cytokinins especially kinetin increases microtubers number because of its positive effect on cell elongation and tuberization (Romanov et al. 2000). In the present study, our finding revealed that the addition of BAP into the nutrient medium was more effective in inducing *in vitro* tuberization in comparison to kinetin. In the later case, tuberization was observed, but in a lower rate. On the other hand, it was observed that tuberization from underground stolons is induced in a higher percentage for all treatments in comparison to the areal stolons. Moreover, the formation of micro and minitubers was observed without the need for a pre-tuberization stage. The obtained results are in accordance to those stated by Liljana et al. (2012) who observed tuberization only in the case of 2 mg/l BAP + 1 mg/l IAA. Meanwhile, Badoni & Chauan (2010) found as highly effective for potato *in vitro* tuberization BAP cytokinin in concentrations up to 10 mg/l. Recently, Vural et al. (2018) in their study on *in vitro* micropropagation of potato used 2.5 mg/l BAP + 0.5 mg/l NAA for microtubers formation.

CONCLUSIONS

On the basis of the obtained results, it can be concluded that the addition of growth regulators i.e., BAP and kinetin in nutrient media is important for enhancing micropropagation coefficient and microtuber rate production of potato cv Vermosh. During *in vitro* multiplication process, there were no observed differences between BAP and kinetin containing media. Meanwhile, regarding *in vitro* tuberization, addition of BAP in the nutrient media was more effective than kinetin. Another important conclusion is the positive correlation between underground stolons and rhizogenesis, callogenesis and tuber formation. The results proved that the technique used in this study can be applicable for *in vitro* multiplication and microtuberization of potato cv. Vermosh and might be a possible for *in vitro* cloning of other potato cultivars.

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