Improvement of Callogenesis Ability by Selecting a Better Hormonal Balance in Potato (*Solanum tuberosum* L).

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Abstract

Response in tissue culture is highly genotype dependent, significant genotypic differences in callus initiation response were observed among both potato genotypes investigated. The Spunta variety develops the best callogenesis in all media compared to Kondor variety depending on hormones concentration, there is a range of variations in days needed for callus initiation, percentage of explants that developed callus, its texture, color and the degree of its formation. Our results show that the callus depends on explants type. Sprout explants respond best to callus formation. The amount of callus ranges from 60 % to 90 % for Kondor and from 60 % to 100 % for Spunta. Callus color after eight week was light green or green yellow for both varieties. The highest amount of callus 100 % was obtained with the combination (NAA x BAP) (0.5/1 mg/l, medium M2) and (2/2 mg/l, medium M3) with Kondor bud explants. In media M1 (1/0.5 mg/l), M2 (0.5/1 mg/l) and M3 (2/2 mg/l) with sprout explants of Spunta the amount of 80 % was noted, callus in media M1 and M2 produced microtubers, shoots and roots.

Keywords: Potato cultivars; callus culture; in vitro; hormonal balance.

1. Introduction

Cultivated potato (*Solanum tuberosum* L) is one of the most important vegetable crops in the world (Solmon-Blackburn & Baker, 2001). Plant regeneration became a useful technique and is applied to solve the problems of many agricultural crops. The Creation of novel germplasm through the techniques of tissue culture and gene transfer holds great potential for improving the quality, resistance to diseases and agronomic characters of potato (Jayaree et al., 2001). Plant regeneration in potato has been progressed a lot in recent years (Shirin et al., 2007). Successful *in vitro* plant regeneration has been achieved from explants of different organs and tissues of potato such as leaf (Cearley et al., 1997), stem (Garcia & Martinez, 1995); (Haque et al., 1996), tuberdiscs (Mozafri et al., 1997); (Esna –Ashari & Villiers, 1998) and unripe zygotic embryos (Pretova and Dedicova, 1992). Callus formation depends on the source of explants, nutritional composition of the medium and environmental factors (Arora & Chawla, 2005). Callus induction and plant regeneration from explants require the presence of appropriate concentrations and combinations of plant growth regulators in the culture media. Auxin is a phytohormone involved in the control of various aspects

of growth and development in higher plants (Rück et al., 1993); (Byale et al., 2013); (Vaishali et al., 2013); (Kaur et al., 2014); (Maryam et al., 2014). The auxin signal is received by plant cells and rapidly transduced to a wide variety of responses in the growth and development of plant organs. These include changes in the direction of growth, shoot and root branching, and vascular differentiation (Leyser, 2001). Much work has been carried out on potato to enhance callus induction, improve the frequency of plant regeneration from the callus and investigate the factors affecting plant regeneration. Different ratios of exogenous auxin and cytokinin determine cell fates in the callus, indicating the importance of these ratios and the potential crosstalk between these two hormones in pattern formation during organ regeneration (Cheng et al.,2012). The main objective of the present study was to investigate the effects of various concentrations of NAA alone and in combination with BAP on callus induction in two variety of potato (*Solanum tuberosum* L).

2. Materials and methods

2.1 Plant material and disinfection

The present work was conducted in the Laboratory of Genetics, Biochemistry and Plant Biotechnology, Constantine, Algeria. Two potato cultivars (Spunta and Kondor) were obtained from the collections of agriculture ministry.

The explants segments (internodes of stem from tubercles one month old and buds existing on potato tubercles), approximately 0.5 cm long were surface sterilized by immersion in 70 % ethanol for 1 min followed by rinsing them in 0.5 % NaClO solution for 15 min and then washed with autoclaved distilled water four five times to remove traces of NaClO. Then, they were transferred to full strength of MS fortified with NAA in combination with BAP (NAA/BAP: M1 (0, 5/1mg/l), M2 (1/ 0,5mg/l) and M3 (2/2 mg/l), and NAA alone M4 (0.5 mg/l), M5 (1.0 mg/l) and M6 (2.0 mg/l). The cultures were incubated at 25 ± 1ºC in a rhythmic cycle of 16 hours light followed by 8 hours darkness was given to the cultures. Light was provided by a 2000 - 3000 lux at 25 ± 1ºC maintaining 60-70 % humidity.

2.2.1 Data collection and statistical analysis

Data were collected by counting the number of callus pieces induced .After eight weeks of incubation, the frequency of callus induction was calculated according to the following formula:

$$\text{Callus induction frequency } \% = \frac{N_{\text{explants produced calli}}}{N_{\text{explants cultured}}} \times 100$$

2.3 Histological analysis

Histological analysis of the internodes calli was performed according to (Boissot et al. 1990). Calli were collected 3 and 60 days after cultured on callus induction medium. Both types of tissues were fixed in FAA (formalin-acetic acid-ethanol) for 24 h. Then, the samples were dehydrated in a graded series of ethanol (70, 95 and 100%) for 1 h each one and embedded in paraffin wax. Samples were cut into 9-12 µm sections and were stained with eosine-alcyan blue and observed under a light microscope and documented using a

digital camera (ET 2760).
The experimental design was a randomized block factorial design with three replicates, and the data was analyzed by computer using a statistical package (STATISTICA program).

3. Results and discussion

Callus initiation on the cut ends of cultured explants in vitro can be observed in all hormone combinations after 3–5 days. The data was analyzed after eight weeks of culture and the result showed that there was a wide range of variations in days for callus initiation, percentage of explants that developed callus; callus texture, callus color and degree of callus formation depending on culture media compositions (Tables 1, 2 and 3, 4).
Calli with Kondor developed roots, stems, leaves and even micro-tubers in the medium M2 and M3 (figure 1 D-E), the finding of (fotso and al. 2014) showed that The ratio NAA/Kin of 0.4/0.4 mg/l combined with 20 to 30 mg/l sucrose was more effective on the microtuberization than NAA and Kin used separately.
As for the degree of development, generally the Spunta variety callus gave the largest callus in media M5 (Figure 1C) and M6 compared to others media. After two months of incubation calli of Kondor have a compact structure with different colors ; green in medium M1, M2 , M4 , M5, M3, brown green and yellowish green in M6. Spunta callus present a compact structure with different colors; green in media M3, brown green in M2, M5 and yellowish green in M1, M6, and M4

3.1 Histological study

A callus consists of an amorphous mass of loosely arranged parenchymatous cells arising from the proliferating cells of the parent tissue. Frequently, as a result of wounding, a callus is formed naturally at the cut end of a stem or root.
The histological study of cal obtained from internode explants after 3 and 60 days chow that the cell division occurred in the epidermis only after three days of culture (Figure 1D), cell division extended to the first layer of the cortex Small protuberances with elongated cells (figures 1C-D).
It consisting of cells which formed as result of divisions became visible on the epidermis and on cut surfaces, The cells of this region are all relatively undifferentiated and meristematic (Figure 1F), densely protoplasmic and with large nuclei and they all undergo active cell division (Figure 1E).
The size of cal increased and become compact after two month figure 2b after the proliferation of friable superficial cal , according to Juan et al.(2012), There were wide intercellular spaces between non embryogenic cell cells and the cells had a very low capability for division.

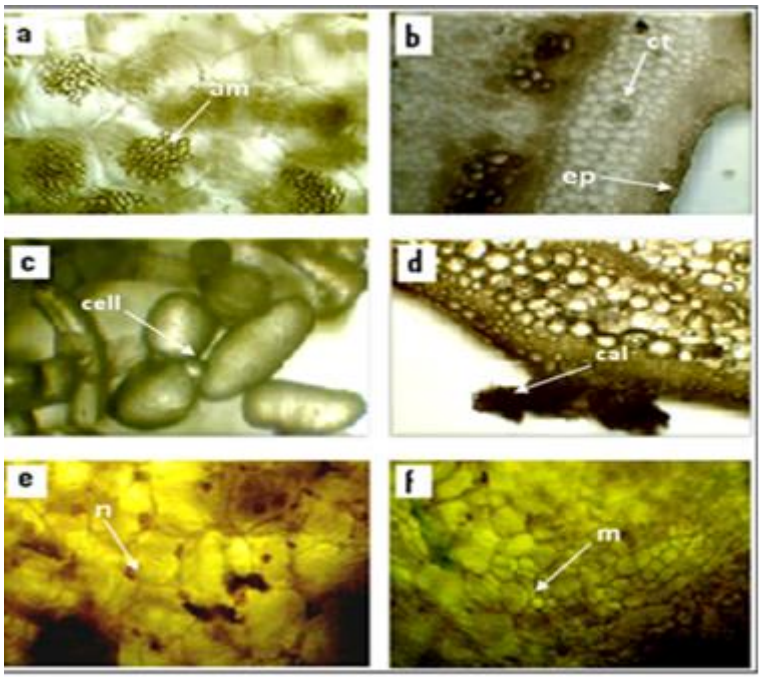

Figure 1. Histological study of the callus of Spunta. a: Cross section of the potato internodes (ct: Cortex, ep: epidermis). b: Shoot tissue (am: Amyloplast). c: Cal initiation from the epidermis cells and cortex. d: Cells shape of cal obtained in figure C (cal proliferation from the epidermis). e: The cells of the callus culture after two months n: nucleus). f: Cal development after 60 days (cal green compact, m: meristematic cells).

3.2 Effect of auxine alone

Auxins favor rhizogenesis and callus formation also influences the same phenomenon in all species. In this investigation, it was observed that auxin when used alone (M4, M5 and M6) produced friable white callus in a shorter period (one week) for both varieties (Figure 2A), and became after eight weeks compact, light green or green yellow (Figures 2B-C). Khadiga et al.(2009) produced green friable callus in one week from potato cultivar using different concentrations of 2, 4-D. The highest degree for Spunta (80 % with M1 and 100 % with M2 and M3) with bud and internodes explants consecutively were recorded.

With the use of NAA alone (Table 3,Figure 2C),the results on callogenesis revealed that whatever is the genotype or the type of explants used, the rate of callus formation of bud explants ranges from 60 % to 100 % in media (M1,M2 and M3) for Spunta and 80% for Kondor for all media. However, for internodes explants, the rate of callus formation is 75% for all media in the case of Kondor and ranges from 30 % to 100 % for Spunta.

This result is in agreement with Shirin et al. (2007) who used 2, 4-D for callus induction from internodal and leaf explants obtained from four potato cultivars including Diamant, and it was found that among all concentrations and combinations 2,4-D at 3.0 mg/l is the most effective auxin concentration for callus induction in all cultivars.

Table 1: Effect of different concentrations of NAA and NAA-BAP on texture and color after after one week and two months callus formation in the case of bud explants with both cultivars.

Hormones concentrations mg/l			**Texture and color after one week**		**Texture and color after two month**	
			Spunta	**Kondor**	**Spunta**	**Kondor**
M1	NAA BAP	0.51	Friable-white	Friable-white	Light- green	Green-yellow
M2	NAA BAP	1 0.5	Friable-white	Friable-white	Light- green	Brown-green
M3	NAA BAP	2 2	Friable-white	Friable-white	brown	green
M4	NAA	0.5	Friable-white	Friable-white	Light- green	Green-yellow
M5	NAA	1	Friable-white	Friable-white	Light- green	Brown-green
M6	NAA	2	Friable-white	Friable-white	Green-yellow	Green-yellow

M1, M2…M6 (culture medium) , NAA (Auxine), BAP (Cytokinine)

Table 2: Effect of different concentrations of NAA and NAA-BAP on percentage of callus formation and day to callus initiation after one week and two months callus formation in the case of bud explants with both cultivars.

Hormones concentrations mg/l		**Percentage of callus formation (%)**		**Day to callus initiation**	
		Spunta	**Kondor**	**Spunta**	**Kondor**
NAA BAP	0.5 1	60	80	Five days	
NAA BAP	1 0.5	100	80		
NAA BAP	2 2	100	80		
NAA	0.5	100	100		
NAA	1	100	60		
NAA	2	60	100		
Mean		100*	84*		
Standard deviation		0	07.48		

* ANOVA (p=0.05) not significant

3.3 Effect of NAA-BAP combination

In vitro cytokinins promote differentiation and multiplication of cells and tissues. Like auxin and gibber-ellin, it may also induce cell enlargement. The results represented in (Table 2) show the influence of the concentration of growth regulators on callus formation in potato. The highest amount of callus (100 %) was obtained with the combinations (NAA / BAP) (1/0.5 mg/l, medium M2) and (2/2 mg/l, medium M3) with

bud explants of Kondor. In medium M1 (0.5/1mg/l), M2 and M3) with bud explants of Spunta, the amount of 80 % was noted.

Furthermore highest amount of callus formation, 90 % for Kondor and 100 % for Spunta in medium M6 were recorded with internodes explants (Table 4). The findings of Shirin et al. (2007) showed that the combination of BAP and NAA at the concentration of 5 and 4 mg/l, respectively, produced maximum callus from nodal explants of potato cultivar.

According to Majid et al. (2014), the NAA in combination with BAP (1.0 mg/l for each) lead to the best degree of callus formation with potato explants after 21 days of incubation. On the other hand Hera et al. (2014) obtained the maximum growth rate and biomass accumulation for *Nigella sativa* with the combination BAP (2 mg/L) + NAA (1 mg/l). Ighilhariz et al. (2008) reported that the use of hormonal combination AIA/ kinetin with various concentrations tested (0.5/0.5 mg/l, 1/1 mg/l and 1.5/1.5 mg/l) induces callus formation from explants of stems and leaves of species, *Atriplex canescens* and A. halimus, with different amounts of callus formation depending the varieties.

Several studies showed that the use of auxin and cytokinin ensures initiation or callus induction in a large number of species: two medicinal plants viz. *Adhatoda vasica* and *Ageratum conyzoides* (Renu and Nidhi, 2011); Sauropus *androgynous* (Udhaya et al., 2012); Atriplex; *Dianthus caryophyllus* L. (Mohamed et al., 2014).

On the other hand (Manel et al., 2014) were obtained highest callus formation (100 %) MS medium supplemented with the combination (NAA/KI=1/1.93 mg/l), for medicinal plant *Calligonum comosum* L. The interactions between BAP and NAA hormones were the most effective factor for callus induction and production. The highest callus production was obtained from 5-days-old seedling in MS medium with BAP (0.5/1 mg/l) and NAA (0.5 /1 mg/l) in *Brassica Napus* (Borjian and Arak, 2013).

Table 3: Effect of different concentrations of NAA and NAA-BAP on Texture and color after one week and two months callus formation on internode explants with both cultivars.

Hormones concentration mg/l			**Texture and color after one week**		**Texture and color after two months**	
			Spunta	**Kondor**	**Spunta**	**Kondor**
M1	NAA BAP	0.5 1	Friable-white	Friable-white	Light- green	Light -green
M2	NAA BAP	1 0.5	Friable-white	Friable-white	Light-green	Light- green
M3	NAA BAP	2 2	Friable-white	Friable-white	Light- green	Green-yellow
M4	NAA	0.5	Friable-white	Friable-white	Light- green	green
M5	NAA	1	Friable-white	Friable-white	Green-yellw	Light-green
M6	NAA	2	Friable-white	Friable-white	Green-yellw	Green-yellow

M1, M2…M6 (Medium culture), NAA(Auxine), BAP (Cytokinine)

3.4 Explants effect

It was found that the callus formation was triggered on all tested explants, whatever was their type (internodes / buds) or genotype (Kondor / Spunta). Our results show that callus formation depends on

explants type. Explants of bud have the best response to callus formation, the amount of callus ranges from 60% to 90 % for Kondor and from 60 % to 100 % for Spunta. We also found that the bud explants has a strong inclination for organogenesis while the stems induce embryogenic callus.

The findings of Qamar et al. (2001) on the kiwi (*Actinidia chinensis*), showed that the callus rate varies according to the type of explants; 97.5 % for the petioles, 94.5 % for inter-node, 88% for leaves and 71% for the meristems.

Variations in callus forming ability of different explants types have been reported in many other plants (Ishii et al., 2004). From the study of different genotypes and potato tissue, including: anther, immature embryos zygotic, stems sections tubers, leaves and roots. Teresa et al. (2005) have found different rates from exhibiting callus indicating that the type of explants influence significantly callogenesis. On other hand Mohammad et al. (2014) show that the callogenesis specificity of explant type would be explained by their differential reactivity to media components.

3.4 Genotype effect

Table 4: Effect of different concentrations of NAA and NAA-BAP on percentage of callus formation and day to callus initiation after one week and two months callus formation in the case of bud explants with both cultivars

Hormones concentrations mg/l	**Percentage of callus formation (%)**		**Day to callus initiation**	
	Spunta	**Kondor**	**Spunta**	**Kondor**
NAA 0.5 BAP 1	75	80	Three to five days	
NAA 1 BAP 0.5	75	30		
NAA 2 BAP 2	75	50		
NAA 0.5	75	70		
NAA 1	66.66	70		
NAA 2	100	90		
Mean	77.77	65		
Standard deviation	11.38*	21.67*		

*ANOVA (p=0.05) not significant

Response in tissue culture is highly genotype dependent. Significant genotypic differences in callus initiation response were observed among the both potato genotypes investigated. The variety Spunta develops the best callogenesis in all media compared to Kondor variety, the findings of Gabriele and al. (2003) on six varieties of Primula show that the rate of induction of callus varied between 24 % and 95 %, which confirms that the genotype is a limiting factor in callus formation. Furthermore Koutoua et al. (2007), worked on seven varieties of durum wheat (*Triticum durum*) and proved that callogenesis depends on the genotype variety.

3.5 Callus development

Depending on hormones concentration, there are variations in the number of days to callus initiation,

percentage of explants developing callus; callus texture, callus color and degree of callus formation (Table 1 and 3).

Callogenesis is initiated after three to five days of culture for both varieties. It is always preceded by a friable white color tissue explants in both varieties and for two types of explants and for all media, the explants loses theirs original characteristics, we noted that cell enlargement and cell division predominate to form an unorganized mass of cells. As a result , the explants undergoes an irreversible changes in its shape, size, and is followed by the appearance of little irregular cellular masses around the cut edges or at the cut edges or from the ruptured surface and upper face, then, the callus becomes greater beyond the seventh day of culture.

Mutasim et al. (2010) produced callus in potato variety in 7-17 days, 100 % of the explants were formed yellow, and watery callus and recorded the highest degree for callus formation. Similar findings were reported by Shirin et al. (2007) and Nistor et al. (2009).

After the 20th day, calli from (sprout) and (internode) presented a compact structure with different colors: green, brown, white. Calli from bud explants of Kondor developed organs (stems, roots and even leaves in media (M1, M2, M3, M4) and Spunta in media (M3, M4, M6).

After two months, calli from bud explants produced microtubers in media (M2, M3, and M4) for Kondor. On the other hand callus from internode can proliferate indefinitely and has little or no organogenesis except for the Spunta variety in media M1 in which it developed small roots.

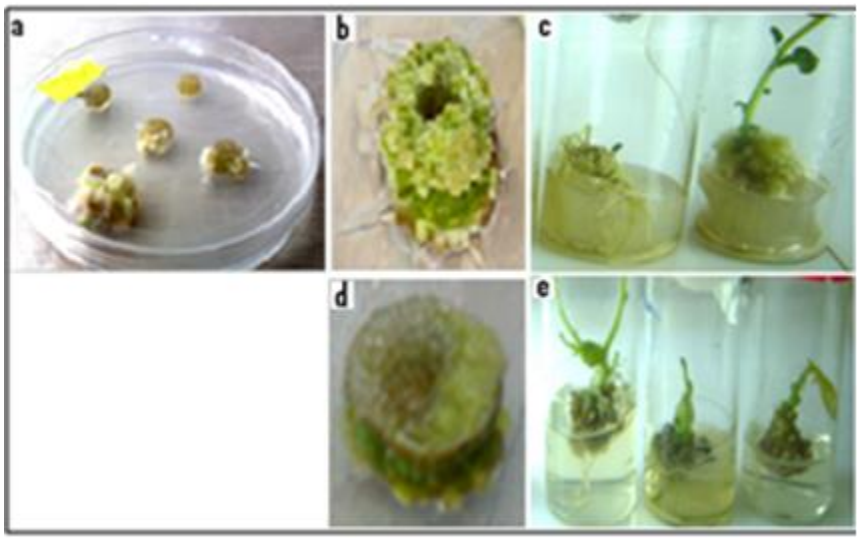

Figure 2. Effect of auxin and cytokinin ratio on callus induction on potato explants (internodes and bud) grown *in vitro*. a: White callus initiation after one week in medium 3 (2/2 mg/l) with Spunta. b: Compact green callus in all surface of internodes explants of Kondor in medium 3 (2/2 mg/l) after 40 days. c: Root and shoot development on Callus of Spunta buds in medium 5(2 mg/l) after 40 days. d: Green Callus in the cut end of internodes explants of Kondor in medium 5(1 mg/l) after 20 days. e: Microtubers formation on the callus of Kondor in medium 1(0.5/1 mg/l) after 40 days.

4. Conclusion

The callus induction was obtained in MS medium supplemented with NAA alone and in combination with BAP. However, best amount of callus formation, 90 % for Kondor and 100% for Spunta in medium M6 was recorded with internode explants .On the other hand the variety Spunta develops the best callogenesis in all media compared to the Kondor variety. Our results show that the callus depends on explants type. Explants of bud have the best response to callus formation, the amount of callus ranges from 60% to 90 % for Kondor and from 60 % to 100 % for Spunta. Callus from bud developed shoot and root in all media, and microtuber in medium (M1 and M2).

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