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Regeneration of pepper (*Capsicum annuum L.*) and evaluation of antioxidant compounds at various ripening stages

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ABSTRACT

A laboratory study was conducted to develop a broadly applicable *in vitro* regeneration method for pepper. Therefore, ten different pepper genotypes were analyzed with regard to their efficiency for regeneration *in vitro*. Cotyledons were used *in vitro* from young seedlings as target tissue for regeneration of pepper. Multiple shoot was induced by culturing cotyledons explants in MS medium supplemented with 0.28 µM IAA and 13.86 µM Zeatin. Results strongly showed that there were significant differences between pepper genotypes. Generally the highest values of all regeneration parameters were obtained from genotypes Balady, Ciliegia piccante and Yellow California wonder. Genotype Hot Horn had the lowest regeneration parameters while other genotypes were moderate response. The rooted shoots were transferred to the greenhouse and showed a normal development to mature plants.

Changes in antioxidant constituents (ascorbic acid, phenolics, and carotenoids) were monitored during three maturity stages in 10 genotypes of pepper. In an attempt to explain the variations during maturity stages (green, intermediate and red/ yellow), the data was expressed on dry weight basis. Ascorbic acid content declined progressively with advancing maturity. Genotype Sigaretta di bergano had the maximum content (2999.0 mg/100 g dry weight) at the green stage. On dry weight basis, phenolic content declined in majority of the genotypes during maturity to red stage. This decline was significant in Ciliegia piccante, California wonder, Sigaretta di bergano and Balady. Genotype Balady and Yellow California wonder had the highest phenolic content of 833.0 mg/100 g and 723.0 mg/100 g, at their final red and yellow maturity stages, respectively. With maturation, total carotenoids and β-carotene content increased significantly of most genotypes. Topepo rosso was a promising genotype in terms of both total carotenoids and β-carotene content. The study proposes the nutritional significance of consuming peppers at the red maturity stage because of enhanced functional properties. Overall genotype Balady and Topepo rosso represent superior genotypes for both nutrition and germplasm improvement.

KEYWORDS | pepper, *in vitro* regeneration, antioxidant compounds, ascorbic acid, phenolics, carotenoids.

Introduction:

Pepper (*Capsicum annuum* L.) is one of the most important vegetables grown in Egypt and many other regions around the world (Ochoa-Alejo and Ramirez-Malagon, 2001; Aboshama, 2011; El Nagar, 2012). To date pepper is used fresh or dried in various foods. Its nutritional properties including antioxidants are important for human nutrition (Mateos *et al.*, 2003; Orlinska and Nowaczyk, 2015). Furthermore, pepper is also a source for natural colors and as medicine (Valadez-Bustos *et al.*, 2009; Zhuang, *et al.*, 2012).

In vitro plant regeneration is essential for the rapid multiplication of disease free planting materials and is an imperative for the application of biotechnology tools to plant breeding and genetic improvement (Christou and Klee, 2004). It is also important for the conservation of genetically pure planting materials. Micropropagation is advantageous over traditional propagation as it can be used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It also leads to simultaneous accomplishment of rapid large-scale propagation of new genotypes (Dabauza and Pena, 2001; Bhagowati and Changkija, 2009). The conventional method of pepper plant propagation using seeds is restricted by the short span of viability and low germination rate of seeds. Lack of natural vegetative propagation in Chili is the limiting factor in conserving genetic purity and micropropagation can be a remedy for it (Raj *et al.*, 2015).

Despite the strong influence of the pepper variety on regeneration frequency, organogenesis has been efficiently achieved from cotyledons (Agrawal *et al.*, 1989; Christopher and Rajam 1996; Venkataiah *et al.*, 2003; El Nagar, 2012; Orlinska and Nowaczyk, 2015). Genotype independent systems for *in vitro* propagation of pepper have still to be established. The developments of such systems remain extremely difficult with the lack of efficient *in vitro* multiplication (Shivegowda *et al.,* 2002).

The direct and positive relationship between health and diet has now attracted the attention of plant breeders and biotechnologists who are directing their efforts to breed genotypes with high content of phytochemicals (Chassy *et al.,* 2006; Kevers *et al.,* 2007; El Nagar and Mekawi, 2014 a and b). Levels of these antioxidants can vary with genotype, stage of maturity, plant part consumed, and conditions during growth and post-harvest handling (Daood *et al.,* 1996; Antonious *et al.,* 2006; Kanner *et al.*, 2006). Thus it becomes pertinent to study these variations in different genotypes during maturity to select the best for health benefits (Marín *et al.,* 2004; Matsufuji *et al.,* 2007).

Peppers, among vegetables, have become extremely popular for the abundance and the kind of antioxidants they contain. Genus *Capsicum* is a rich source of phenolics (Howard *et al.*, 2000; Blanco-Ríos *et al.,* 2013). Peppers are considered an important source of ascorbic acid, therefore, they have been attributed health benefits (Howard *et al.*, 1994; Kumar and Tata, 2009). Their attractive colors are due to the profuse synthesis of various carotenoid pigments during ripening (Hornero-Mendez *et al.,* 2000). The knowledge of the changes, occurring during growth and maturation, holds great significance from both dietary and nutritional point of view. It is therefore, imperative to study the changes in the content of antioxidants as influenced by different genotypes and their maturity stages. There are also very few research have been carried out in

identifying and compare the ascorbic acid content, total Phenolic compound, and carotenoids contents among different varieties of green pepper with different maturity stages (Zhang 2003 ; Deepa *et al*., 2007 ; Helmja *et al.*, 2007; Kumar and Tata , 2009 ; Leja *et al.,* 2008; Iqbal, 2009 ; Medina-Juárez *et al*., 2012; Shaha *et al*., 2013 ; Castro-Concha *et al*., 2014). The aim of the present work was to develop a broadly applicable *in vitro* regeneration method for pepper and to evaluate different genotypes of regenerated peppers, harvested at different maturity stages for their antioxidant content.

Materials and Methods:

The present experiment was conducted in the Biotechnology Laboratory, Research Park, and in the Vegetable farm, Faculty of Agriculture, Benha University, Egypt to evaluate the total phenolic compounds, total flavonoid compounds and carotenoids content in fruits obtained from *in vitro* regeneration of ten pepper genotypes.

Plant material:

Mature seeds of ten pepper genotypes were used to raise seedlings for the present study. Seeds of the local genotype Balady, Sigaretta di bergano, Piccante di cayenne, Annheim M, Ciliegia piccante, Topepo rosso, California wonder , Yellow California wonder , Hungarian sweet wax and Hot horn were obtained from the Preservation Germplasm Laboratory of the Department of Horticulture, Faculty of Agriculture, Benha University, Egypt. The first 7 genotypes had red fruits at ripening stage while the last three genotypes had yellow fruits.

Establishment of aseptic plants:

For establishing aseptic cultures of pepper growing *in vitro*, dry mature seeds were surface sterilized according to method described by El Nagar, 2012. Sodium hypochlorite a common disinfectant surface sterilize plant tissues was used. Seeds of the pepper genotypes were immersed in a 2.5% sodium hypochlorite for 10 min which is present in commercial bleach solutions (Clorix). Then they were rinsed five times with sterile distilled water for 10 min each. During immersion and rinsing the solution was stirred on a shaker at 200 rpm under the laminar air flow hood. The sterilized seeds were placed into sterile tissue culture jars (30 seeds/jar) containing a half concentrated basal MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg *et al.,*1968), 3.0% sucrose and solidified with 0.7% Oxoid-Agar. The medium was adjusted to pH 5.8 before autoclaving at 121°C and 1.2 kg/cm2 to 1.3 kg/cm2 pressure for 20 min. All cultures were incubated at 25°C ±1°C under florescent light (2000 LUX) and a 16 h photoperiod.

***In vitro* plant regeneration:**

Seeds of pepper were grown aseptically for providing starting material. According to the standard procedure described by El Nagar 2012, explants of cotyledon of ten pepper genotypes were taken from aseptic plants 10 days old after *in vitro* germination of the seed. Cotyledons divided into 1-cm pieces and cultured horizontally on the MS medium supplemented with 0.57 µM IAA, 22.81 µM Zeatin and 10.0 µM $AgNO_3$ for 4 days. Thereafter, shoot induction media (MS medium supplemented with 0.28 µM IAA and 13.86µM Zeatin) were used to induce shoot proliferation by culturing 10 explants each in petri dishes (60 x 15 mm). If clusters of organogenic tissue, shoot buds, or single shoots emerged, they were harvested and transferred to 250 ml sterile tissue culture containers for elongation (MS media contained 0.27 µM NAA and 9.12 µM Zeatin). Those cultures were transferred to fresh medium every second week. For rooting and adaptation, shoots with a length of at least 2 cm were transferred to root induction medium (½ MS-medium). Finally, they were trained in the greenhouse. Plants grew to maturity, producing flowers and fruits. Seed viability from ripening fruits was tested in soil in the greenhouse and on germination medium *in vitro*.

All peppers received similar water and fertilizer treatments. Fruits of ten pepper genotypes were harvested at the same time but at three successive maturity stages viz. green (fruits showed characteristic green colour), intermediate (50% of the fruit showed transition from green to red/yellow) and finally at full maturity stage (bright red/yellow colour depending on the genotype). Immediately after harvest, the fruits were placed in polyethylene bags and were then stored at - 20 °C until analyzed. Quantitative analysis was carried out for ascorbic acid, total phenolic, total carotenoids and β-carotene contents.

Ascorbic acid content:

Ascorbic acid was quantitatively determined according to 2,6-dichlorophenolindophenol-dye method as described by Jones and Hughes (1983) with slight modifications. The ascorbic acid in 3 g of dry sample was extracted with a 3% meta-phosphoric acid (v/v). The extract volume was made up to 100 ml, mixed and centrifuged at 3000 g for 15 min at room temperature. Ten milliliters were titrated against standard 2,6-dichlorophenolindophenol dye, which was already standardized against standard ascorbic acid. Results were expressed on mg /100 g dry weight.

Total phenolic content:

Total soluble phenols, in ethanol extracts, were determined with Folin-Ciocalteau reagent using the method of Slinkard and Singelton, 1997. Dry peppers (2 g) were thoroughly powdered and homogenized in 10 ml of 80% ethanol containing 1% HCl. The homogenate was placed in capped test tubes and heated at 60 °C in a water-bath for 60 min. This step helps to complete the extraction of the phenolics as well as to destroy ascorbic acid to a large extent. The reducing property of ascorbic acid has been shown to interfere in estimation of phenols by Folin's reagent. The extract was cooled and centrifuged at 10,000 rpm for 15 min at 4 °C. The resulting supernatant was collected and the pellet re-extracted and the supernatants were pooled together. The final extract was concentrated in a flash evaporator and the volume reduced to 20 ml. The same extract was used for the estimation of total phenolics. Results for phenolics were expressed as mg /100 g dry weight catechol equivalent.

Total carotenoids and β-carotene:

The extraction of carotenoids was carried out according to the method described by Mınguez-Mosquera and Hornero-Mendez (1993). A known weight dry samples were milled in a coffee grinder and 2 g of obtained powder sample was extracted with acetone in mortar and pestle. Extractions were repeated until the complete exhaustion of colour (usually 4–5 extractions were enough). All extractions were pooled in a separating funnel and shaken with diethyl ether. A sufficient quantity of 10% NaCl was added at the end to facilitate separation of the two phases. Aqueous phase was discarded. The lipophilic phase was washed with 100 ml of an anhydrous Na-2SO4 (2%) solution to remove all the remaining water. It was saponified with the addition of 40 ml of 10% KOH in methanol and shaken vigorously before being left in a dark place for 1 h. After addition of water, the pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator and then made up to 25 ml with acetone. One milliliter aliquot of this solution was centrifuged at 12,000 rpm and stored at -20 °C until analysed. Losses occurring during the process were monitored with the use of all-transb-apo-80-carotenal as internal standard. All analysis was carried out in triplicate. Because of no availability of standards for different carotenoids, total carotenoids were estimated by taking the absorbance of extracts at 450 nm (Ranganna, 1986). However, the separation and quantification of β-carotene was carried out by the method of Chávez-Mendoza (2013) using C18-type column (Hypersil ODS C18) of 4.6 mm × 15 cm. Chromatographic analyses were carried out using a young lin HPLC, series YL-9100, equipped with a quaternary pump, an autosampler (YL9150), a degasser, and a YL-9160 spectrophotometric detector (Photo Diode Array detector–PDA), which was set at 455 nm. The solvent system consisted of acetonitrile/THF/H2O (85:12.5:2.5). The analyses were made at 24 °C. The final results were expressed as mg/100 g dry weight *Capsicum* tissue.

Experimental design and Statistical analysis:
Experiments were arranged in a completely randomized block design with 3 replications. Data were estimated as the mean and its standard error of the different traits. The calculations were done using Microsoft Excel 2010 program.

Results:
***In vitro* regeneration of pepper:**
To establish the tissue cultures, aseptically grown seedlings were used. For the evaluation of the germination capacity, seeds of the pepper genotypes were observed *in vitro* (Figure 1). Differences were observed between genotypes after ten days of culture on germinating agar medium. The genotypes California wonder, Yellow California wonder and Sigaretta di bergano showed the highest germination frequency (Table 1). Their germination rates were 93.0%, 90.0% and 86.0%, respectively. The lowest rate was observed in genotypes Hot horn with 25.0% and Piccante di cayenne 30% germination (Table 1). The other genotypes were moderate in their germination frequency 54.0% to 74.0% (Table 1).

To investigate the *de novo* shoot induction from cotyledons, regeneration rates of ten pepper genotypes were compared. The percentages of explants forming shoots, elongated shoots, rooted shoots as well as numbers of mature plants were recorded. Explants of all pepper genotypes studied regenerated shoots from cotyledons on shoot induction medium (Figure 1). Genotypes California wonder and yellow California wonder regenerated shoots at highest rates of 85.0% or 70.0%, respectively, whereas from genotypes Hungarian sweet wax and Anaheim M only 15.0% or 17.0% formed shoots, respectively (Table 1). Explants of all pepper genotypes initiated multiple shoots. Genotypes California wonder (hot) and Balady developed the highest number of shoots per explant of cotyledon with a mean value of 5.1 and 4.6 shoots, respectively. The lowest percentage of shoot formation was observed for genotype Hot horn (2.0 shoots per explant). Balady following by Ciliegia piccante genotypes were the best of shoot elongation as well as rooted shoots (Table 1). Successfully rooted plants generally grew well under greenhouse conditions. Using our optimized procedure, we repeatedly regenerated mature pepper plants from 10 different *Capsicum* genotypes (Table 1). On average, explants developed in less than 9 months to mature, flowering, and fertile plants (Figure 1).

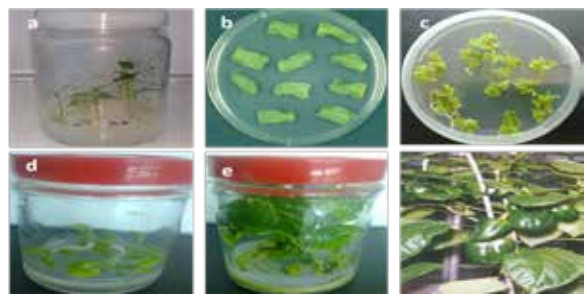

Figure 1: Plant regeneration from cotyledons *of Capsicum annuum* cv. Balady.
(a) Seed germination (b) Explants of cotyledons on shoot induction medium (c) Formation of shoots from cotyledons explants 4 weeks after cultured on MS medium supplemented with 0.28 µM IAA and 13.86µM Zeatin (d) Elongated shoots on shoot elongation medium (e) Rooting pepper plantlet (f) Normal regenerated pepper plants.

Table 1: Comparison of the regeneration frequencies of ten pepper genotypes during different stages of *in vitro* cultivation.

Genotypes	Germination of seed (%)	Induced shoots (%)	Mean ± SE of shoots per explant	Elongated shoots (%)	Rooted shoots (%)	Mature plants (%)
Balady	64	33	4.6±0.3	59	90	88
Sigaretta di bergano	86	60	3.5±0.2	30	70	69
Piccante di cayenne	30	20	3.0±0.1	25	56	55
Anaheim M	54	17	2.3±0.4	20	50	45
Ciliegia piccante	82	55	4.1±0.3	49	85	80
Topepo rosso	74	45	2.6±0.2	22	55	53
California wonder	93	85	5.1±0.3	40	70	66
Yellow California wonder	90	70	4.4±0.2	45	61	58
Hungarian sweet wax	69	15	2.1±0.2	15	43	39
Hot Horn	25	23	2.0±0.1	16	37	36

Ascorbic acid:
Ascorbic acid content was found to vary significantly among pepper genotypes harvested during different maturity stages. The content on dry weight varied from 1000 mg /100 g in Piccante di cayenne to 2999 mg/100 g in Sigaretta di bergano at the green stage. At the red stage it ranged from 700 to 2101 mg/100 g in the same genotypes (Table 2). This depicted a 3 fold variation in ascorbic acid content among genotypes. At the green stage, genotypes Sigaretta di bergano (2999 mg /100 g) and Balady (2708 mg /100 g) had significantly higher content than the rest (Table 2). Sigaretta di bergano also had the highest content (2101 mg /100 g) at the red stage followed by Anaheim M, Balady and yellow California wonder. Ascorbic acid content showed a declining trend with advancing maturity.

Total phenolic content:
Total phenolic content was measured by using Folins reagent. Although, it overestimates the total phenolics due to interfering compounds such as ascorbic acid, it is so far the only single and widely used method for estimating total phenols. However, necessary corrections were employed for ascorbic acid interference, as described in materials and methods. The results when described on dry weight indicated that total phenolic content in the different genotypes of green peppers ranged from as low as 192 mg /100 g in Hot Horn to 1112 mg /100 g in Balady, depicting a 6 fold variation between genotypes (Table 2). The content in red peppers varied from 326 mg/ 100 g in California Wonder to 833 mg /100 in Balady, depicting a 2.5 fold variation. Balady was the single genotype with significantly high levels of phenolics at all the three stages of maturity (Table 2). Yellow California wonder also had significantly high phenolic content of 723 mg/100 g at the yellow stage. However, during maturity from green to red stage, a declining trend was observed in four genotypes viz., Ciliegia piccante, California wonder, Sigaretta di bergano and Balady whereas Piccante di cayenne and Topepo rosso showed no significant differences. Yellow genotypes namely, Hungarian sweet wax, Hot Horn and Yellow California wonder exhibited an increasing trend.

Table 2: Ascorbic acid and total phenolic content in peppers during different maturity stages.

Genotype	Ascorbic acid content (mg/100g DW)			Total Phenolic Content(mg/100g DW)		
	Green	Intermediate	Ripening stage	Green	Intermediate	Ripening stage
Balady	2708	2257	2005	1112	970	833
Sigaretta di bergano	2999	2101	2101	503	392	392
Piccante di cayenne	1000	750	700	498	490	480
Anaheim M	2003	2051	2073	295	322	363
Ciliegia piccante	1990	1202	1202	487	475	403
Topepo rosso	1255	1204	1199	503	503	503
California wonder	1802	1507	1106	422	415	326
Yellow California wonder	1803	2002	1506	432	625	723
Hungarian sweet wax	1508	1257	1257	374	248	396
Hot horn	1801	1506	1104	192	184	360

Carotenoids content:
Total carotenoid content (dry weight) ranged from 8 mg/100 g in Balady to 45 mg/100 g in Topepo rosso at the green stage. There was sharp increase in carotenoid content with maturity and at the red stage Topepo rosso showed the highest content of 134 mg/100 g while Balady had the least (13 mg/100) (Figure 2). Thus with advancing maturity a maximum of 3 fold variation was observed in genotype Topepo rosso (Figure 2). β-carotene content (dry weight) ranged from a lowest of 502 mg /100 g in Balady to 3101 mg/100 g in Ciliegia piccante at the green stage (Figure 3). At the red stage it ranged from 901 mg/100 g in Sigaretta di bergano to 6202 mg/100g in Anaheim M. Appreciably, high content (3705, 4501, 5199 and 6003 mg/100 g) was also observed in Ciliegia piccante, Balady, Topepo rosso and California Wonder at the red stage. Yellow genotypes, namely Hungarian sweet wax, Hot horn and Yellow California wonder had low content ranging from 1501 to 2102 mg/100 g. β-carotene content increased with advancing maturity. Significant differences in β-carotene content with respect to different maturity stages were observed in genotypes, Topepo rosso, California wonder, Anaheim M, Balady and Yellow California wonder (Figure 3). Dramatic increase (13 fold) in β-carotene content was observed in Balady, during maturity (Figure 3).

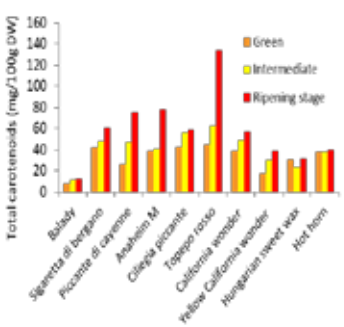

Figure 2: Total carotenoids content in peppers during different maturity stages.

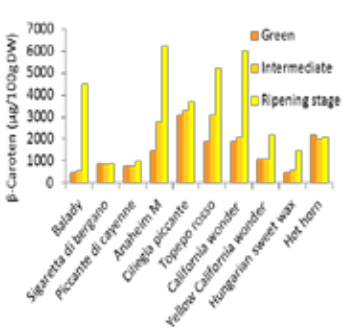

Figure 3: β-carotene content in peppers during different maturity stages.

Discussions:
The low efficiency of plant regeneration by pepper cultures has been widely reported (Steinitz *et al*., 1999; El Nagar 2012; Orlinska and Nowaczyk, 2015). *In vitro* plant regeneration is the result of interplay of factors related to the genotype used as the source of explants, and the culture medium which is important for the expression of this capacity. To facilitate *in vitro* regeneration of pepper, it was necessary to optimize these factors to routinely regenerate whole plants.

In most studies explants of cotyledons were chosen to initiate tissue culture from *C. annuum* L. (Wolf *et al*., 2001; El Nagar, 2012). Therefore, in the present study cotyledons from ten days old seedlings were excised and used as explants. Their regeneration frequency was recorded. The percentage of explants of cotyledons forming shoots ranged from 15.0% (Hungarian sweet wax) to 85.0% (California wonder). Multiple shoots emerged from cut ends of cotyledons. The mean number of shoots per explant ranged from 2.0 to 5.1 shoots depending on the genotype. The proportion of shoot elongation ranged from 15.0% (Hungarian sweet wax) to 59.0% (Balady). Elongated shoots were able to form roots. The percentage of root formation ranged from 37.0 % to 90.0%. Differences of shoot formation shoot elongation and rooting from explants of cotyledons could be due to genotypic differences. In addition, the the size of the surface wound may influence the vigor of cell division (Christou and Klee, 2004). Many researchers reported that the percentage of shoots forming from explants of cotyledons were 0.0% to 100.0%. The average number of shoots per explant ranged from 0.0 to 6.2, but very few of these shoots elongated (Agrawal *et al.,* 1989; Arroyo and Revilla 1991; Szasz *et al.,* 1995; Christopher and Rajam 1996; Berljak 1999; Dabauza and Pena 2001; Venkataiah *et al.,* 2003; El Nagar 2012).

Depending on the genotype we found previous that the use of zeatin increased shoot formation capacity 12.0% to 84.0%. These data confirmed earlier observations in peppers from Binzel *et al*., 1996; Shivegowda *et al*., 2002). Therefore, in the current study the IAA combined with zeatin were used for shoot formation from pepper genotypes.

Ascorbic acid:
It is difficult to compare the results of different workers because the changes during different maturity stages have been expressed on fresh weight instead of dry weight (Howard *et al*., 2000; Marin *et al*., 2004). Variation in ascorbic acid content could be accounted to the changes in the moisture content in peppers during different maturity stages (Deepa *et al*., 2007). Therefore, it is important that for evaluation of changes occurring during maturity within a genotype, the antioxidant content calculations be based on dry matter as it will clearly establish whether the obtained results are due to metabolic effects or otherwise.

Factors that may influence to the ascorbic acid content are the genetic and environmental factors (Howard *et al.,* 1994; Antonious, *et al.* 2006). Chassy *et al*., 2006 found that genotype

has the greatest influence on the level of phytochemicals in fruits and vegetables. Marín *et al*. 2004 and Matsufuji *et al*. 2007 reported higher ascorbic acid content in other pepper cultivars. Medina-Juárez *et al*., 2012 and Zhuang *et al.,* 2012 reported that the ascorbic acid content of peppers is mainly dependent on the cultivars. For these reasons, in this study, the genotype is the factor that may have greater impact on the ascorbic acid content because the environmental factors and growth conditions were the same for all cultivars.

Results in table 2 showed that a gradual increase of ascorbic acid content from green to intermediate stage while, decreased in the lateral stages. These results were agreed according to the data reported by Kumar and Tata, 2009. Blanco-Ríos *et al*., 2013 found that green bell pepper contained the most amount of ascorbic acid, followed by the red and yellow bell peppers. The red and yellow cultivars were not significantly different.

Total phenolic content:
Peppers are an important source of total phenols, which are mainly localized in the peels (Zhang and Hamauzu, 2003). In general, data on changes in antioxidant constituents are usually expressed on fresh weight and expression on dry matter is ignored. Therefore it becomes necessary to establish comparison on dry weight in order to ascertain whether the obtained values are due to moisture loss or metabolism effects (Deepa *et al*., 2007). Keeping this in view, all the data in the text has been expressed especially on dry weight. The values observed here in current pepper genotypes were considerably low in comparison to those reported by Howard et al. (2000) in *Capsicum spp*. Varietal differences, may have accounted for higher values. Our results on changes during maturity are in line with Howard *et al*., 2000 who observed an increasing trend in the total phenolics during maturity in majority of *Capsicum* cultivars. On the contrary, Marin *et al.*, 2004 have shown a marginal decrease in total phenolic content during maturity from green to red stage. Peppers contain a very rich polyphenol pattern, which includes hydroxycinnmates, flavonols and flavones (Marin *et al.,* 2004). Thus for a clear understanding of metabolic changes in phenolics during maturation it is necessary to characterize the phenolic profile.

It is well known that content of phytochemicals, including phenolic compounds present in vegetables, is affected by the specie and type of pepper, agronomic conditions, maturity, postharvest handling and pre and postharvest treatments applied to the fruit (Howard *et al*.,2000; Iqbal, 2009). Vinson *et al.* (1998) and Sun *et al.* (2007) reported lower levels of total phenols in Bell peppers than those found in the present study. Contrarily, Helmja *et al.,* 2007 reported a higher content of these compounds in pungent pepper (480 mg/100g fresh weight). Similarly, Kevers *et al.* (2007) reported higher levels of total phenols in red, yellow and green peppers (296, 284 and 215 mg/100 g, respectively), even higher than those found in spinach, broccoli, cucumbers and carrots. Medina-Juárez *et al.,* 2012 found that high levels of total phenols in all the pepper extracts studied. Caribe pepper presented the highest value and Jalapeno pepper the lowest. Blanco-Ríos *et al.,* 2013 found that green bell pepper had the highest total phenol content, and no significant differences between red, yellow, and orange were observed. Shaha *et al.,* 2013 reported that a gradual increase of phenolics concentration was observed from green to red ripening.

The accumulation of bio active compounds is determined by factors internal to the organism (genotype), but it can be strongly modified by the conditions of the growing season (Mateos *et al.,* 2003). However, environmental factors contributing significantly to the differences among cultivars can be minimized when the fruits are grown in semi-controlled conditions. Therefore, the differences in the phenolic levels of the studied pepper genotypes were mainly due to genetic differences because the conditions of growth for the ten cultivars analyzed were similar. The genetic differences of each cultivar can lead to differences in the biosynthetic pathways and fruit composition (Hornero-Méndez *et al.,* 2000; Leja *et al.*, 2008).

Carotenoids content:
Peppers are also a good source of carotenoids, which can vary in composition and concentration owing to differences in genetics and maturation (Russo and Howard, 2002). During ripening of peppers the green colour due to chlorophyll and carotenoids such as lutein disappear with the synthesis of chromoplast pigments (Hornero-Mendez *et al.,* 2000). However, comparison between genotypes revealed a maximum variation of 11folds between Topeppo rosso and Balady at the red stage. These values are low in comparison to 690–1320 mg/100 g dry weight reported in 5 red *Capsicum* fruits by Hornero-Mendez *et al.,* 2000. This is in line with previous reports quantifying pepper carotenoids as a function of maturity (Howard *et al.,* 2000; Zhang and Hamauzu, 2003). In the report by Marin *et al.,* 2004, β-carotene content has been observed to be 1.7–4.3 mg/100 g fresh weight basis in pepper cv. Vergasa. However, Daood *et al*., 1996 have reported a similar increase in β-carotene content at the last stages of ripening. Besides, the authors have also highlighted the significant differences found between the different cultivars with regard to the antioxidant vitamin content.

Blanco-Ríos *et al.,* 2013 found that red cultivar had the highest content of total carotenoid, while green cultivar showed the lowest. Orange and red peppers contained the highest level of β-carotene, followed by the yellow pepper; while the green cultivar had the lowest level. In contrast, Sun *et al*., 2007 found higher contents of β-carotene in the green and red cultivars of bell pepper. These differences could be attributed to the different weather and growing conditions prevailing in the two studies. The higher or lower carotenoid content for a given cultivar depends on various factors: greater or lesser expression of the genes governing carotenogenesis, physiological and morphological characteristics intrinsic to the cultivar, and growth conditions (Hornero-Méndez *et al.,* 2000; Kanner *et al.,* 2006). The last factors can be ignored in the present study because the conditions of growth for the ten cultivars analyzed were similar.

Conclusion:
The present study demonstrates a simple and promising protocol for *in vitro* plantlet regeneration of *C. annuum* L. from cotyledon explants. The use of IAA in combination with zeatin favored plant regeneration. This protocol can be applied for the conservation and multiplication of genetically pure and disease free genotypes and also for transgenic experiments for enhancing nutrional values in *Capsicum annuum* L. Significant variation in total phenols, carotenoids, and ascorbic acid content between pepper genotypes and maturity stages, indicates that the potential efficiency of antioxidants vary considerably with both genotypes as well maturity stages. Nutritionally, peppers at the red stage are a good source of mixture of antioxidants including ascorbic acid, carotenoids and polyphenols. Genotypes, Anaheim M, Balady and Topepo rosso have rich dietary composition, which may offer potential health benefits.

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