Influence of cytokinin and explant type on *in vitro* culture of three pepper (*Capsicum annuum* L.) hybrids

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Abstract

Pepper (*Capsicum annuum* L.) is an important vegetable crop grown worldwide and propagated commercially by seeds. It is well known that pepper was recalcitrant to tissue culture techniques and several studies have shown that the response was genotype -dependent. In order to improve the regeneration capacity of three Libyan pepper hybrids namely, Ziad×254, Ziad×38 and Nizar×24, a study was conducted to clarify the effects of cytokinin combination; Benzyl adenine (BA) at 4, 5 or 6 mg/L combined with Kinetin (Kin) at 1 mg/L, and type of explants; shoot tips and single nodes (1-1.5 cm long) on *in vitro* proliferation. Explants were excised from 10 days old *in vitro* grown seedlings and cultured onto MS medium supplemented with cytokinins. The cultures were incubated at 25 ± 2ºC under continuous 16-h photoperiod (40 µmol m^{-2} s^{-1}) using cool white fluorescent lamps. Results indicated that BA + Kinetin at 5+1 mg/L led to high number of shoots per explant and higher number of leaves per growing shoot. Shoot tips explants were superior to single nodes based on the studied parameters. A slight difference was observed in shoot proliferation in the three hybrids. Rooting was 100% after 7 days of culture by using IBA at 0.5 mg/L.

Key words: *Capsicum annuum*, Cytokinins, *in vitro* culture.

Introduction

Pepper (*Capsicum annuum* L.) is among the most economically important vegetable crops grown worldwide and propagated commercially by seeds. In Libya, pepper is considered as one of the most widely grown undercover vegetables (FAOSTAT, 2012). The cost of hybrid seeds is so high along with the lack of specialized personnel to perform the necessary work needed for pure lines and hybrid seed production, which necessitates the establishment of a micropropagation protocol for production of uniform plants, that if succeful, will reduce the dependence on seed propagation.

It is well known that pepper was recalcitrant to tissue culture techniques and several authors have shown that the regenerative capacity was strongly influenced by genotype (Ochoa-Alejo & Ireat-Moreno 1990, Christopher & Rajam 1996, Mock & Norzulaani 2007). Organogenesis from many explants has been described such as hypocotyle (Gunay and Rao 1978) cotyledon (Dong Zhaolong *et al.* 2003) shoot tips explants (Kumar *et al*. 2005, Asraffazzaman *et al*. 2009) .

In Libya, no published reports on *in vitro* micropropagation of locally produced pepper inbred lines or hybrids, and in order to establish a micropropagation protocol, the present work was conducted to test the effect of cytokinins and type of explants on *in vitro* multiplication of three local peppers cultivars namely; Ziad×254, Ziad×38 and Nizar×24.

Materials and Methods

Explant preparation- Three hybrid pepper cultivars produced in a local breeding programme namely; Ziad×254, Ziad×38 and Nizar×24 were used in this study. Surface sterilization of seeds was performed by soaking the seeds in running tap water for 15 min. with agitation, then rinsing in 70 % ethanol for 1 min followed by immersion in 2.5 % Clorox (4.5% active

chlorine) for 15 min. Seeds were rinsed with sterile distilled water and were cultured on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 3% sucrose and gelled with 0.7% Agar without growth regulators for germination (fig. 5 A). The cultures were incubated at 25 ± 2ºC at 16 hrs in cool white fluorescent lights. Explants; shoot tips and single nodes (1-1.5 cm) were excised from 10 days old seedlings and used for the experiments.

Culture media- To test the effect of cytokinin combination and type of explants on shoot regeneration, shoot tips and single nodes were cultured onto MS medium supplemented with BA at 4, 5 or 6 mg/L combined with 1 mg/L Kinetin. Shoots grown form shoot tip explants were subcultured into MS medium contained BA at 5 mg/L for another four weeks. For rooting, growing shoots excised from the cultured explants were cultured singly into culture tubes containing MS medium supplemented with IBA at 0.5 mg/L. After 3 to 7 days in culture rooting was evaluated visually, and plantlets were transferred into glass jars with the same medium composition for further growth (fig. 5 B and C). All cultures were incubated under the above mentioned conditions. The number of leaves and shoots formed were recorded. Plantlets were acclimatized and transplanted onto soil mix consisted of sand and peat moss at 1:1(v/v).

Data collection and statistical analysis- Observation were made on the number of growing shoots per explant, number of leaves per shoot, percent rooting (%), and number of roots per plantlet after 4 weeks of culture. Shoot tips were subcultured onto the same medium for another 4 weeks and data were recorded accordingly. The experiment was a factorial experiment consisted of three cytokinin combinations as the main plot and type of explants as a subplot and genotypes as sub-subplot. Treatments were assigned in a completely

randomized design with 10 replicates. Data were subjected to analysis of variance (ANOVA) using SAS software and the means were separated using Duncan's multiple range test at 5% probability level.

Results and Discussion

It was evident that benzyl adenine (BA) significantly affected number of shoots per explant and number of leaves in the growing shoots (Fig.1). BA at 5.0 mg/L (44.4µM) led to a significant increase in the number of leaves and number of growing shoots as compared to 4.0 and 6.0 mg/L. Successful micropropagation depends on using the appropriate type and concentration of growth regulators. Cytokinins such as benzyl adenine was used singly or in combination with other cytokinins or auxins for micropropagation of peppers cultivars and the recommended level used was 5.0 mg/L combined with other growth regulators (Agrawal *et al.* 1989, Sanatombi & Sharma 2007). Several authors have used benzyl adenine, Kinetin or Thidiazuron (TDZ) for pepper regeneration (Mok & Norzulaani 2007). Sanatombi and Sharma 2007, used BA at 44.4 µM (5.0 mg/L) with Kin at 4.6 µM (1.0 mg/L) and achieved high response in *Capsicum fruitscens*.

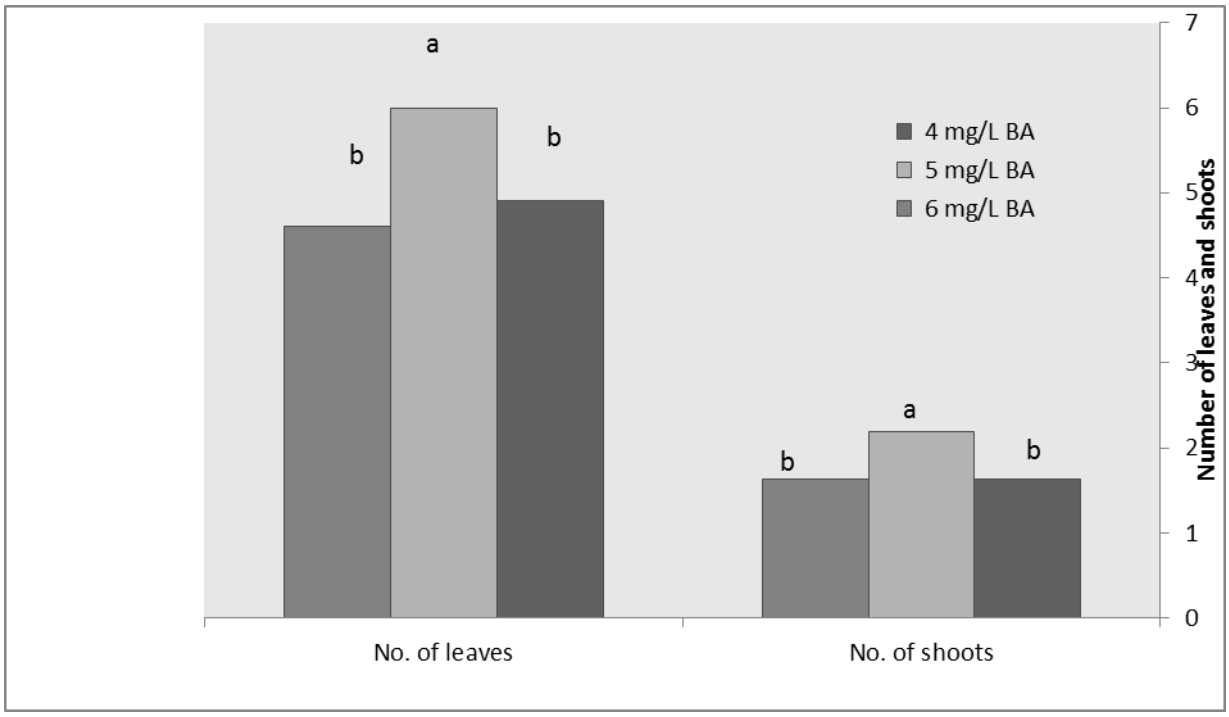

Fig. 1. Effect of Benzyl adenine at 4.0, 5.0 or 6.0 mg/L combined with Kinetin at 1.0 mg/L on number of shoots and number of leaves after four weeks of culture.

Type of explants significantly affected the number of growing shoots and number of leaves per shoot (Fig.2). Shoot tips were superior to single nodes as they produced the highest number of shoots and leaves. Benzyl adenine at 5.0 mg/L was more effective in both types of explants in achieving the highest number of growing shoots and leaves. Several authors used shoot tips or single nodes in pepper micropropagation as they maintain genetic fidelity of the resulted plants (Kumar *et al*. 2005, Sanatombi & Sharma 2007, Asraffazzaman *et al*. 2009).

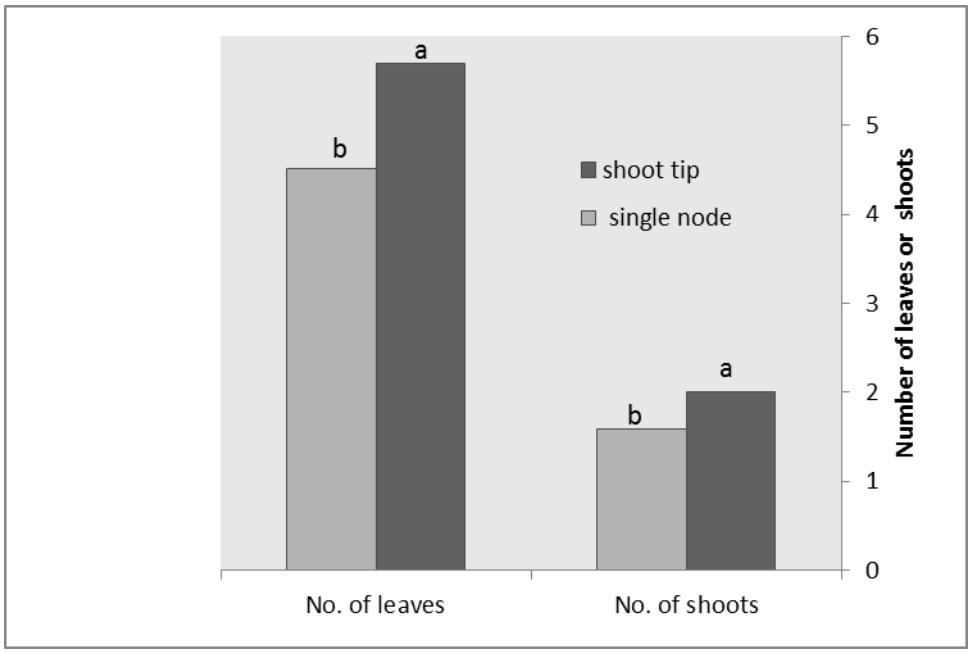

Fig. 2. Effect of explants type on number of shoots and number of leaves after four weeks of culture.

Pepper genotypes responded well *to in vitro* culture, however a slight diffreneces were observed regarding the number of shoots and number of leaves (Fig.3). Nizar×24 had significantly higher number of shoots than Ziad× 38 and Ziad× 254, and had the highest

number of leaves per shoot. Genotypes were shown to have a strong influence on regeneration of pepper (Ochoa- Alejo & Ireta-Moreno 1990, Christopher & Rajam 1994), moreover, the ability of specific explant to regenerate in culture varies with the species and cultivar (Ebida & Hu 1993, Christopher & Rajam 1994, Szász *et al*. 1995).

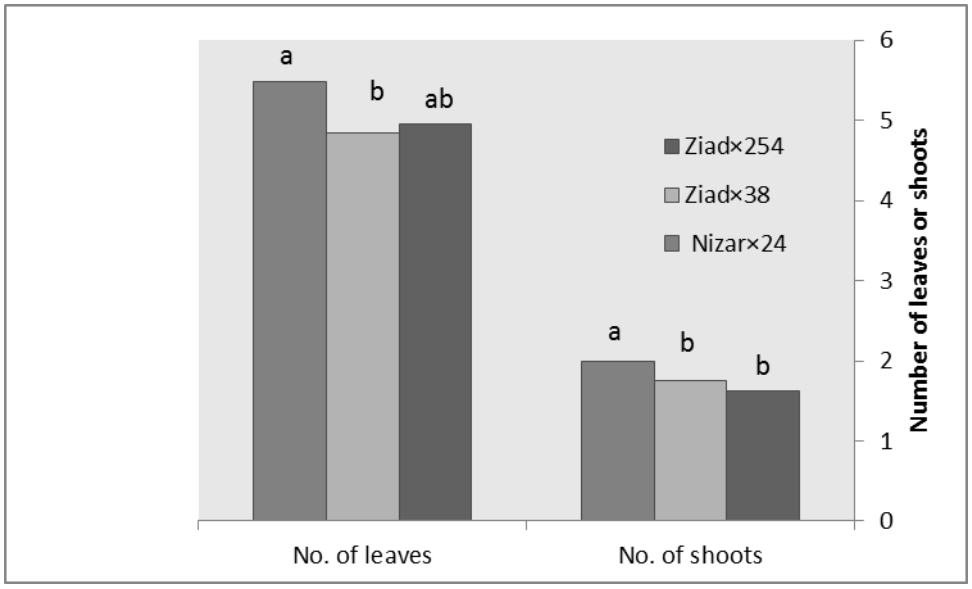

Fig. 3. Effect of genotypes on number of shoots and number of leaves after four weeks of culture.

Rooting - Shoot tip explants cultured on MS containing; Indole -3-butyric acid (IBA) at 0.5 mg/L started to form adventitious roots apparently, on the third day of culture, and reached 100% after 7 days of culture (Fig. 5B and 5C) in the three hybrids (data not shown). A significant difference was observed in number of root per plant as Ziad×38 and Nizar×24 had significantly higher number of roots than Ziad×254; however no significant differences were observed in number of leaves per plant among genotypes (Fig. 4). The results were in agreement with published reports which used auxins such as IBA at 2.4 µM or IAA at 2.8 µM (Sanatombi & Sharma, 2007) or NAA at 1.0 mg/L (Hussain *et al*. 1999) for rooting of micro-shoots.

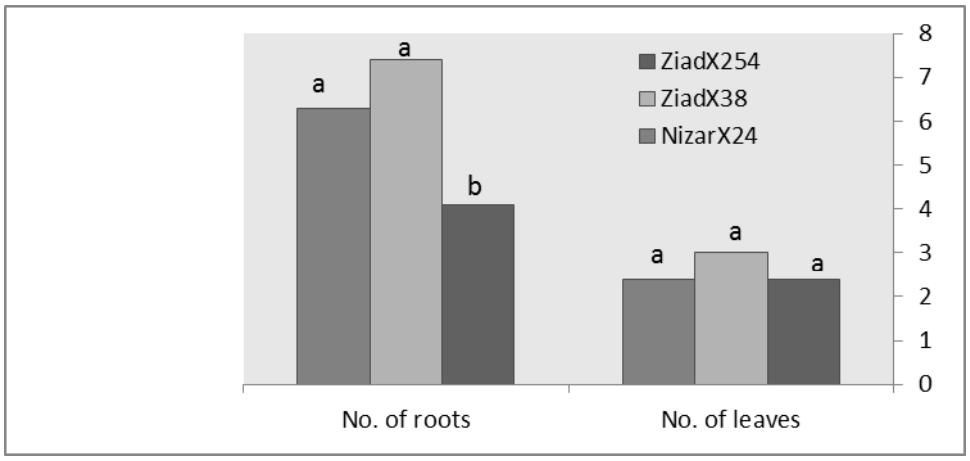

Fig. 4. Rooting of pepper genotypes from shoot tips explants cultured on MS medium with IBA at 0.5 mg/L.

This is the first study on micropropagation of local pepper hybrid that may help to achieve micropropagation of selected pure lines and hybrids. Further research is needed to improve micropropagation of such important hybrids. In conclusion, the results indicated that for multiplication of these three pepper hybrids, BA can be used at 5.0 mg/L combined with 1.0 mg/L kinetin. Moreover, shoot tip explants where more responsive than single nodes. Rooting was 100% using 0.5 mg/L IBA. The three hybrids had a similar trend to *in vitro* culture.

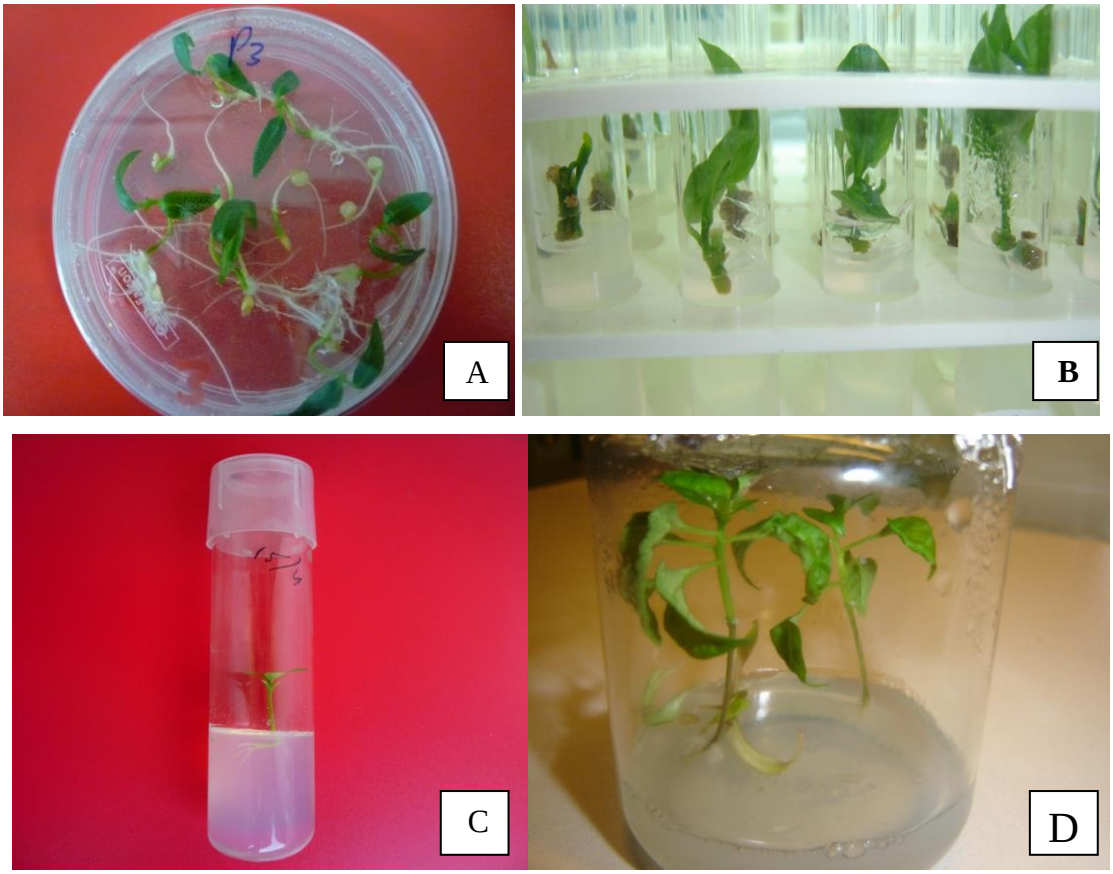

Fig. 5. *In vitro* germination of pepper seeds (A), single nodes growing in culture (B),rooting of shoot tip explants after 3 days (C), and after 3 weeks of culture (D) using 0.5 mg/L IBA in Ziad×254 hybrid.

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تاثير نوع السيتوكينين ونوع المستأصل على الاكثار الدقيق لثلاثة

هجن من الفلفل (*Capsicum annuum* L.)

الملخص العربي

اجريت هذه الدراسة من اجل تحسين القدرة على التوالد لثلاثة هجن ليبية من الفلفل وهي Ziad×254 ، Ziad×38 و Nizar×24 . درس تاثير توافيق من السيتوكينينات بنزايل ادينين (benzyle adenine) بتركيز 4 ، 5 و 6 ملجم/لتر مع الكينتين (Kinetin) بتركيز 1.0 ملجم/لتر، وكذلك نوع المستأصل، القمة الخضرية والعقد المفردة (بطول 1.0 – 1.5 سم) على معدل التضاعف. استخدمت مستأصلات من باذرات بعمر 10 ايام تم انلاتها في البيئة الغذائية وزرعت على وسط موراشيج وسكوج (Murashige & Skoog) المزود بمنظمات النمو تحت ظروق التحضين 16 ساعة ضوء ودرجة حرارة 25±2 م° وشدة اضاءة 40 ميكرومول/م^2/ثانية . اشارت النتائج الى ان بنزايل ادينين والكينتين بتركيز 5 و 1 ملجم/لتر معا حققا اعلى معدل لعدد النموات الخضرية لكل مستأصل واعلى عدد للاوراق لكل نمو خضري. كما وجد ان القمة الخضرية استجابت بشكل افضل من العقد المفردة في الخصائص المدروسة، وقد لوحظ اختلاف طفيف في استجابة الهجن الثلاثة لهذه العوامل. حققت النموات الخضرية نسبة تجذير 100% بعد 7 ايام من الزراعة باستخدام اندول حامض البيوتريك (Indole butyric acid) بتركيز 0.5 ملجم/لتر.