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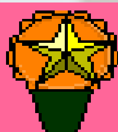

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Full Length Research Paper

Seed salt-soluble protein expression as marker of local *Medicago ciliaris* populations adapted to highly salted region from Algeria (Oran Great Sebkha)

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The present study estimated the diversity of seed storage salt-soluble proteins of Algerian populations of *Medicago ciliaris* species, for understanding biochemical and molecular features involved in response during plant stress adaptation. Eleven patterns of *M. ciliaris* populations with two moderate sensitive ecotypes to NaCl, two reference genotypes and seven prospecting populations near and far from a strongly salted area (Sebkha of Oran) were investigated by one dimensional electrophoresis SDS-PAGE. The results show that the proteins profiles were very similar with 10 to 12 bands profiles about all populations, after electrophoresis in denaturing conditions; but an 80 kDa band was visible only on population from Sebkha origin, where the salt content in the soil is the highest one. It appears that, this globulin protein is related to salt tolerance and could be used as SDS-AGE markers for differentiating between tolerating and sensible *M. ciliaris* populations to salt stress.

Key words: Oran Great Sebkha, annual *Medicago ciliaris*, seed storage salt-soluble proteins, sodium dodecyl sulfate- poly acrylamide gel electrophoresis (SDS-PAGE) markers.

INTRODUCTION

The Oran Great Sebkha is formed by a slender stratum of water devoid of vegetation inside. It is located at 35°32'N, 00°48'E. Lands bordering this site are occupied by private grounds, used for farming. The edges of salty lake are used by breeders for the pasture. Before, Sebkha which was not the object of study on vegetation does not seem to contain a remarkable flora. It remains a halophile vegetation compound of *Sueada sp.*, *Juncus sp.* and small bundles of *Chamaeropsis humilis*, and some rare specimens of tamarisk at the level of bands (Moussa and Saint-Martin, 2011). Presently, observation on the vegetal species is enabling the drawing of the map of vegetation growing around the Sebkha and shows the relationship with salinity fluctuation. Annual medics (*Medicago*

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Abbreviations: MW, Molecular weight; **HCA,** hierarchical cluster analysis**; Rf,** relative mobility; **SDS-PAGE,** sodium dodecyl sulfate- poly acrylamide gel electrophoresis; **UPGMA,** unweighted pair group method with arithmetic means.

Table 1. Name, origin, experimental code and genotype of the different studied populations of *M. ciliaris.*

Pop	Experimental code and genotype name	Province and Origin	Latitude	Longitude	Altitude (m)
FA pop	P1: *M. ciliaris modj1*	Mascara Algeria	35°33'24.29''N	0°72'.86E	689
	P2: *M. ciliaris modj2*	Mascara Algeria	35°24'0.64''N	0°7'10.06E	600
	P3: *M.* ciliaris *modj3*	Bredeah Algeria	35°34'60''N	0°51'0''W	110
	P7: *M. ciliaris modj7*	Ain-Tassa Algeria	35°37'22.00''N	0°55'36.25''W	300
NE pop	P4:*M. ciliaris modj4*	Oran Great Sebkha Algeria	35°33'28.88''N	0°50'33.95''W	81
	P5:*M. ciliaris modj5*		35°33'26.83''N	0°50'32.63''W	84
	P6:*M. ciliaris modj6*		35°33'58.73''N	0°50'28.00''W	83
Ref pop	P8:*M. ciliaris 252*	ITGC Algeria	3501N	0018W	470
	P9:*M. ciliaris 255*	ITGC Algeria	3501N	0018W	470
	P10:*M. intertexta.ciliaris IG54229*	Lebanon ICARDA Syria	33 52N	3601E	1000
	P11:*M. intertexta.ciliaris IG54230*	Lebanon ICARDA Syria	33 52N	3601E	1000

spp.) are predominantly selfing annual plant of the Mediterranean region (Lesins and Lesins, 1979). They are winter annual legumes which indicate a strong ability to adapt to local environments. These species are of special interest, since they form symbioses with nitrogen-fixing bacteria and are, therefore, excellent candidates for the low-input improvement of marginal or degraded lands with low fertility. They are critical components of natural ecosystems and agriculture and have recently been the subject of several studies highlighting their benefits and responses to cultivation in saline conditions (Abdelly et al., 2011). Among these legume species, *M. ciliaris (L.)* appeared to be more salt tolerant within a collection that included *Medicago polymorpha*, *Medicago truncatula* and *Medicago minima*, since it maintained its biomass productivity when growing in 100 mM NaCl (Abdelly et al., 1995). *M. ciliaris* species would have strong potential to be used in the reclamation of areas, such as Sebkha edges. Based on the fact that they were one of the glycophyte plants that have possibility to grow under this salt condition and were competent to improve the quality and quantity of pasture.

M. ciliaris L. is a diploid species (2n=16) belonging to the section of *Spirocarpos* subsection of *Intertextae* as delimited by Heyn (1963). Understanding the genotypic variation for salt adaptation is a key for developing selection and breeding strategies. The main strategy used over the past few years to improve salt tolerance in legumes has been genotype screening and selection (Cordovilla et al., 1995).

Thus, the objective of many studies was to explore genotypic variation for salt tolerance using many indicators of plant's health. Nevertheless, the seed storage proteins associated with ecological data of this species could be very promising. They are useful tools for a preliminary investigation of genetic diversity of local populations of *M. cilaris L.* In addition, Seed proteins are classified according to their solubilities either as water-soluble albumin, salt-soluble globulin, alcohol-soluble prolamin, and acid-or alkaline-soluble glutelin. Seed storage proteins include mainly globulins in legumes. They are the dominant storage proteins in legume seeds and account for 50 to 90% of seed proteins (Rashidah et al., 2007). The expression of seed storage proteins is under strict developmental regulation and represents a powerful model system to study the regulation of gene expression during plant development (Mehrotra et al., 2009).

Present study deals with comparison of three prospecting populations of *M. ciliaris* growing near the Oran Great sebkha and four others far from it. In this way, we examined the expression of seed salt-soluble proteins from different populations of *M. ciliaris* using the cryo precipitation technique for extraction and SDS-PAGE for separation. This information could then be used to define selection criteria and provide a framework for selecting large numbers of populations that grow in all sides of Sebkha edges.

MATERIALS AND METHODS

Plant material

The material included 11 populations of *M. ciliaris*; tow ecotypes were represented on the moderate sensitive ecotypes to salt stress from ITGC Algeria; seven were prospected near and far from the Great Sebkha of Oran and two reference exotic genotypes (*Intertexta vir. Mcilaris*) were supported from ICARDA, Syria. They are formed in three groups of populations Table 1; faraway population (FA pop), nearest po pulation (NE pop) to Oran Great Sebkha and the reference population (Ref pop).

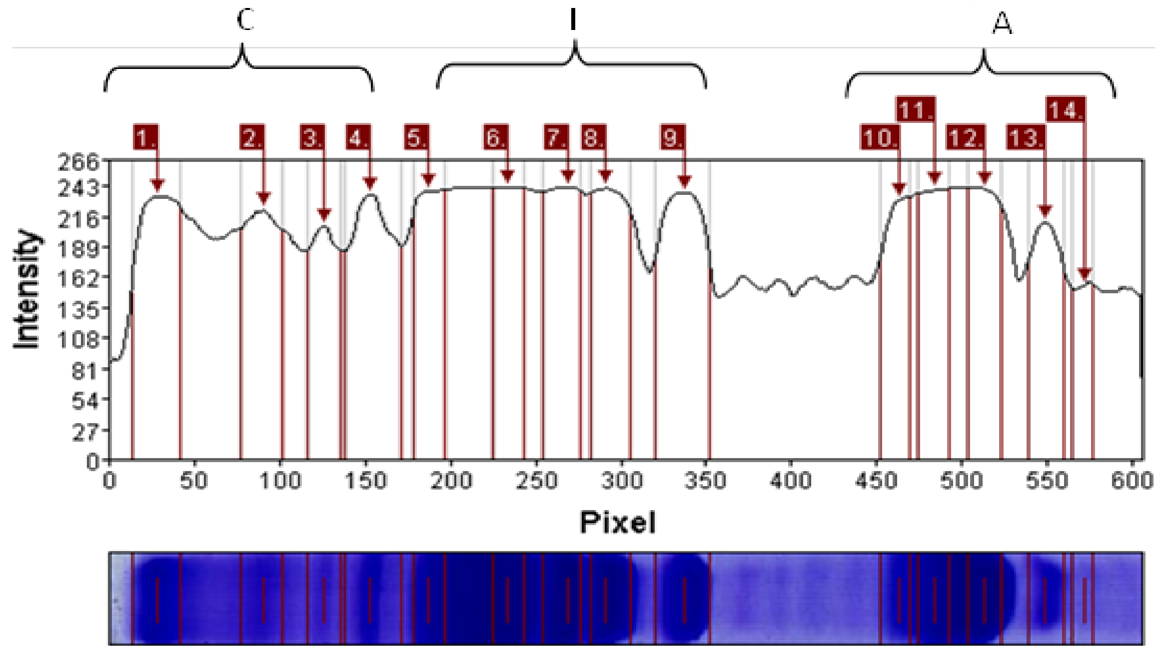

Figure 1. Profile windows content based on software Gel Analyzer V.2010a of the reference genotype P10 (*M. intertexta. ciliaris IG54229)* with the three zones C, cathodic zone; I, intermediary zone and A, anodic zone. 1, 2, 3…14: indicated band number.

Protein extractions

Mature seeds were used for isolation seed salt-soluble proteins. Ten seeds of each population were ground in four volumes of 1-butanol with mortar and pestle to eliminate lipids. Protein fraction was extracted by cryo-precipitation (Anisimova et al., 1991 modified by Fyad-lameche, 1998).

Electrophoretic analysis (SDS-PAGE)

One-dimensional SDS-PAGE (4.5% stacking gel and 13.5% resolving gel) was performed according to Laemmli (1970). The electrophoresis was performed for 4 h at 150 V constant. The gels were stained with Coomasie brilliant blue R250 and then destained in methanol/ acetic acid. After destaining, the electrophoretic bands were clearly visible. The electrophoresis was repeated 4 times. Biolabs Protein Ladder was used as molecular weight marker. It is a mixture of 12 recombinant, highly purified proteins, which resolve into clearly identifiable sharp bands from 10 to 250 kDa (Laemmli, 1970; Sambrook et al., 2001). The 25 and 80 kDa bands have triple the intensity of the other proteins and serve as reference indicators (unpublished results).

Statistical analysis

A comparison of electrophoresis bands was performed based on their thickness, their number and their mobility. The protein ladder is intended for use as a precise size standard when performing SDS-PAGE to calculate the molecular weight of a protein of interest. To avoid any ambiguity, the experience was repeated 4 times and the gels were analyzed using the computer software Gel Analyzer V.2010a. Only the major and clearly bands in the gels were considered for data recording. The data was recorded as presence (1) or absence (0) of protein bands, entered in a binary matrix and then analyzed by statistical procedures. Hierarchical cluster analysis (HCA), similarity and distance matrix were conducted using DendroUPGMA (http://genomes.urv.cat/UPGMA/) applying Jaccard's coefficient with default settings (Garcia-Vallvè et al., 1999).

RESULTS AND DISCUSSION

All profiles were analyzed according software Gel Analyser based on band intensity curves. Using one profile (P11) of the two reference genotypes *M. intertexta vr.ciliaris* (Figure 1), three different zones were recorded (Figures 1 and 2); one is cathodic zone (C), the second is intermediate (I) and the last is anodic zone (A). Each zone contained the major and minor bands. According to the protein ladder, their molecular weights ranged between 60-250, 15-50 and 10-≥15kDa, respectively. In addition, it was observed that protein profiles of most of the populations were the same for the major bands. There are many common bands among populations. But specific bands were also observed, it was indicated by a dark arrow (Figure 2).

The most number of bands belonged to (NEpop) populations with important intensity and profiles with least number of bands with low intensity were related to those of (FApop) distant populations to the Oran Great Sebkha. They presented 15 and 13 bands, respectively. A specific band of 80 kDa with the Rf 0.162 was present in all nearest populations (NEpop) and it was missing in the

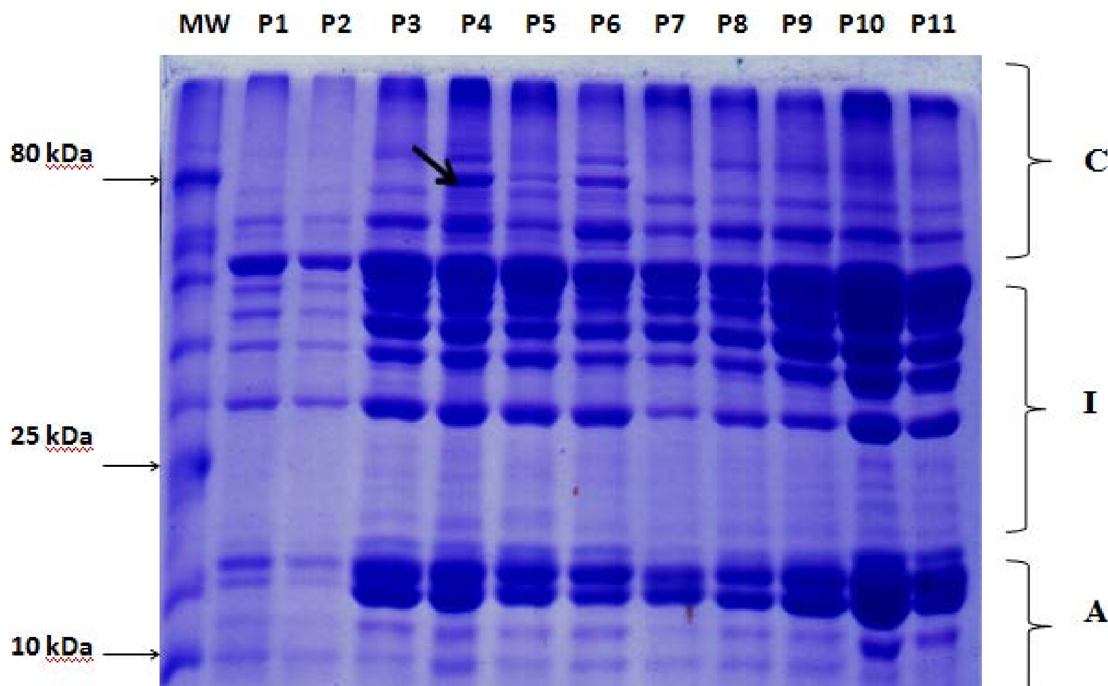

Figure 2. SDS-PAGE of seed salt-soluble proteins of *M. ciliaris* populations with Lanes: MW, Ladder protein (standard protein molecular weight markers); P1, *M. ciliaris modj1;* P2, *M. ciliaris modj2;* P3, *M.ciliaris modj3;* P4, *M. ciliaris modj4;* P5, *M. ciliaris modj5;* P6, *M. ciliaris modj6*; P7, *M. ciliaris modj7;* P8, *M. ciliaris 252;* P9, *M. ciliaris 255;* P10, *M. intertexta. ciliaris IG54229* ;P11, *M. intertexta. ciliaris IG54230 and three zones of migration:* C, cathodic zone; I, intermediary zone; A, anodic zone.

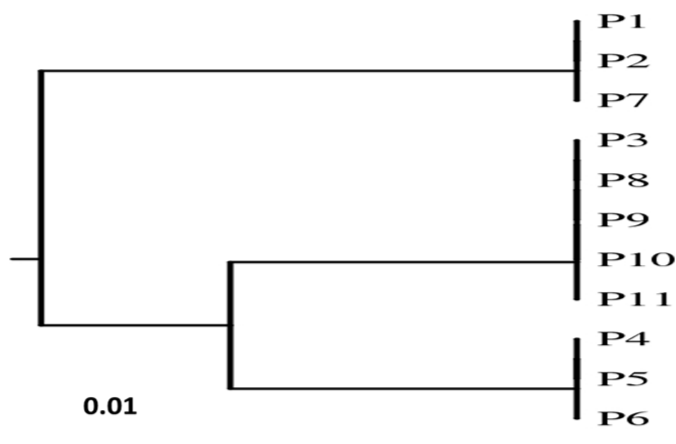

Figure 3. UPGMA dendrogram based on binary matrix of seed salt-soluble protein bands of *M. ciliaris* populations.

distant (FApop) and reference populations (Ref pop). Fareghi et al. (2007) studied the variability between 18 genotypes of Lucerne using SDS-PAGE. They reported 16 bands related to salt-soluble proteins.

Based on cluster analysis by UPGMA method (Figure 3) populations were classified into three groups. The first group consisted of 3 genotypes (P1, P2 and P7), the second group included five genotypes (P3, P8, P9, P10 and P11) and the third group consisted three genotypes (P4, P5, P6). Similarity and distance matrix based on Jaccard coefficient were shown in Tables 2 and 3. The highest similarity and minimum genetic distance belonged to each intra groups with similarity coefficient 1. The lowest similarity and highest genetic distance were observed between first group and third group with similarity coefficient 0.867. They are the distant and close populations to the Oran Great Sebkha, respectively. These two last groups have important similarity with reference group, moderate genetic distance with similarity coefficient 0.933 and 0.929, respectively.

These results confirmed the output dendrogram demonstrated with Phylip format:(((P1:0.000,P2:0.000):0.000,P7:0.000):0.051,(((((P3:0.000,P8:0.000):0.000,P9:0.000):0.000,P10:0.000):0.000,P11:0.000):0.033,((P4:0.000,P5:0.000):0.000,P6:0.000):0.033):0.018); According the output dendrogram, the three groups were clearly separated (Figure 3).

In addition, we observed that the distant populations to Oran Great Sebkha were differed than the tow moderate sensitive ecotypes *M. ciliaris* 252 and 255 by the low intensity of band, although variation was observed in the density or sharpness of a few bands. This indicates that, the salt-soluble protein fractions, such as globulins, should be used for studying the relationship of the seeds

Table 2. Similarity matrix computed with Jaccard coefficient for seed salt-soluble proteins of studied populations of *M. ciliaris.*

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
P1	1	1.000	0.929	0.867	0.867	0.867	1.000	0.929	0.929	0.929	0.929
P2		1	0.929	0.867	0.867	0.867	1.000	0.929	0.929	0.929	0.929
P3			1	0.933	0.933	0.933	0.929	1.000	1.000	1.000	1.000
P4				1	1.000	1.000	0.867	0.933	0.933	0.933	0.933
P5					1	1.000	0.867	0.933	0.933	0.933	0.933
P6						1	0.867	0.933	0.933	0.933	0.933
P7							1	0.929	0.929	0.929	0.929
P8								1	1.000	1.000	1.000
P9									1	1.000	1.000
P10										1	1.000
P11											1

Table 3. Distance matrix based on Jaccard coefficient.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
P1	0	0.000	0.071	0.133	0.133	0.133	0.000	0.071	0.071	0.071	0.071
P2		0	0.071	0.133	0.133	0.133	0.000	0.071	0.071	0.071	0.071
P3			0	0.067	0.067	0.067	0.071	0.000	0.000	0.000	0.000
P4				0	0.000	0.000	0.133	0.067	0.067	0.067	0.067
P5					0	0.000	0.133	0.067	0.067	0.067	0.067
P6						0	0.133	0.067	0.067	0.067	0.067
P7							0	0.071	0.071	0.071	0.071
P8								0	0.000	0.000	0.000
P9									0	0.000	0.000
P10										0	0.000
P11											0

and their environmental origin. Previously, Hedrick et al. (1976) demonstrated, that the genetic variation between and within populations is considered as a result of the environmental heterogeneity and the action of natural selection. Presently, Lazrek et al. (2009) investigated the genetic diversity of a collection of annual *Medicago* species and found that, it had a correlation with environment salinity of origin of these populations. Recently, Amouri et al. (2014) demonstrated that the tolerant genotype to salt stress of annual *Medicago* represented maximum number of total protein bands and the sensitive corresponded minimum number of polypeptide bands using SDS-PAGE.

Our results concluded that the material prospected near and far from the Oran Great Sebkha exhibited moderate genetic diversity for one dimensional SDS-PAGE. Therefore, 2D-electrophoresis is needed to separate various portions of the gel. This later technique has already been used by Li et al. (1998) and they reported their usefulness. These techniques can be applied to studies of storage proteins in other seeds as well as non-seed storage proteins. Krochko and Bewley (1998) used the two-dimensional electrophoresis to determine the composition of seed storage protein fractions in alfalfa. They found that the major seed storage proteins in alfalfa are medicagin (a legumin-like globulin), alfin (a vicilin-like globulin) and a family of lower molecular weight (albumins). These comprise 30, 10, and 20%, respectively; of the total extractable protein from mature seeds. Kaviani and Kharabian (2008) demonstrated that alterations in salt (fertilizers) levels in soil could change the subunits of seed protein legumes.

In general, seed storage protein profiling based on SDS-PAGE can be employed for various purposes such as varietal identification, biosystematics analysis, determination of phylogenetic relationship between different species, generating pertinent information to complement evaluation and passport data (Sammour, 1991). Our results corroborate these objects because we found that seed protein fractions are power tool to identify the adapted populations to Sebkha edges and we can use this substantial protein fraction as molecular markers.

Furthermore, seed proteins are mainly storage proteins and are not likely to be changed in dry mature seed; their

composition is highly stable and is affected only slightly by environmental conditions or seasonal fluctuations (Ladizinsky and hymowitz, 1979). Previously, numerous studies in the last decade have shown that intrinsic changes in the plant such as chromosomal rearrangements or even doubling of chromosome numbers have no, or very small, effects on seed protein profile. The seed storage proteins are encoded by small fraction of the genes expressed in seeds and their expression is regulated at transcriptional, post-transcriptional, translational and post-translational levels (Mehrotra et al., 2009). A precise knowledge about seed protein genes, their regulatory mechanisms and the proteomic approaches can help us to resolve a part of relationship between grain salt-soluble proteins and the adaptation to salt conditions.

Conclusion

This study demonstrates that populations from Oran Great Sebkha edges seem to express a higher level of protein production than distant p, the more expression of seed salt-soluble proteins. It may be concluded that hybridization between populations from the two groups is suggested to be conducted with the expectation that band 80 KDa and other minor bands might be linked with salt stress adaptation. This would help in planning experiments for marker assisted breeding in *M. ciliaris*. The obtained results also allow us to direct the choice of the sites of collections for the future prospecting and the choice of parents according to the fixed objectives (crossing, selection, gene candidates etc).

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Effects of calcium gluconate and ascorbic acid on controlling shoot necrosis during micropropagation of primocane-fruiting raspberry (*Rubus idaeus* L.) cultivars

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***In vitro* shoot necrosis is a quite widespread disorder affecting raspberry micropropagation. This study was conducted to investigate effects of calcium gluconate and ascorbic acid on shoot necrosis and dieback of raspberry shoots during micropropagation. Nodal segments of primocane-fruiting raspberry cultivars 'Allgold', 'Erika', and 'Polka' when cultured on Murashige and Skoog (MS) medium containing 0.6 mg·L^{-1} 6-benzyladenine (BA) and 1 g·L^{-1} calcium gluconate, showed lower explant browning and shoot necrosis and resulted in higher shoot initiation rate in all three cultivars. Ascorbic acid, at 50 and 100 mg·L^{-1}, increased fresh weight of microshoots of all three cultivars. Although culture medium containing calcium gluconate was found to reduce shoot growth and multiplication of 'Allgold' and 'Erika' compared to control, an addition of 1 g·L^{-1} calcium gluconate into MS medium containing 0.6 mg·L^{-1} BA, at shoot induction stage, is recommended to prevent explants browning and shoot necrosis during raspberry micropropagation.**

Key words: Explant dieback, nodal culture, shoot multiplication, tissue culture.

INTRODUCTION

Raspberries (*Rubus idaeus* L.) are traditionally propagated using adventitious buds that arise laterally on cold-treated root cuttings. In the 1980's, micropropagation was introduced as an option in raspberry propagation (Privé and Sullivan, 1991). The use of tissue culture propagation has increased by years as it can produce disease-free plants with rapid and uniform growth. The micropropagated plants spread faster and produce enhanced vegetative growth promoting early establishment and production in the field (Debnath et al., 2012).

However, the *in vitro* establishment of raspberry cultivars is still problematic (Wu et al., 2009). One of the most important problems in raspberry micropropagation is the rapid browning, necrosis, and dieback of the

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Table 1. Medium compositions tested in raspberry micropropagation.

Medium	Medium supplement substances	
	Calcium gluconate ($g \cdot L^{-1}$)	Ascorbic acid ($mg \cdot L^{-1}$)
Control	0	0
1	0.5	0
2	1	0
3	0	50
4	0	100

explants. These problems are partly caused by oxidation of polyphenols which are abundant in raspberry (Mederos-Molina and Trujillo, 1999) and/or by calcium deficiency that occurs during shoot initiation of culture (Abousalim and Mantell, 1994). Other reasons of shoot necrosis during micropropagation include drop of pH of the culture medium, prolonged subculture, and low temperature for the shoot growth (De Block, 1990).

Raspberries are rich in phenols and the phenolic content varies among cultivars (Liu et al., 2002). Phenolic substances, especially oxidized phenols, generally have negative effect on plant micropropagation (Arnaldos et al., 2001). These phenolic compounds are responsible for browning of the culture medium followed by subsequent lethal necrosis of explants (Ozyigit, 2008). The darkening or browning of the medium in tissue culture is caused by exudation and oxidation of phenolic compounds which results in the formation of quinones which are highly reactive and toxic to plant tissues (Ko et al., 2009). Ascorbic acid has been known as a phenol exudation inhibitor (Gil et al., 1998) and used as a medium supplement to decrease the symptoms of necrosis or browning leading to dieback of explants in various plant species (Ko et al., 2009). Ascorbic acid acts as an antibrowning agent in tissue culture by inhibiting the formation of strongly-oxidizing quinones and by inhibiting the activity of phenolases (Chikezie, 2012).

Another problem associating with browning and necrosis of shoots during *in vitro* culture is calcium deficiency. Calcium deficiency in plant can result from limited uptake of calcium ion and inadequate transport, because calcium is not remobilized or non-translocatable (Hepler, 2005). Smaller amount of calcium supply during *in vitro* culture is caused by low transpiration rate due to high humidity in the culture vessel and can introduce undesirable anions.

Addition of supplemental calcium into culture medium has been proved to decrease the necrosis and dieback of explants in micropropagation of some plant species. Calcium chloride added into MS (Murashige and Skoog, 1962) medium in range of 50-100 $mg \cdot L^{-1}$ efficiently prevented necrosis and dieback of shoots of banana and plantains (Martin et al., 2007). De Block (1990) suggested the use of calcium gluconate instead of calcium chloride or other calcium salts to increase calcium levels in the medium without changing the concentrations of other ions. Calcium gluconate, an organic form of calcium, effectively reduced frequencies of shoot tip necrosis in pistachio shoot cultures although it did not prevent the occurrence of symptoms (Abousalim and Mantell, 1994).

The objective of this work was to evaluate the effectiveness of ascorbic acid and calcium gluconate in eliminating tissue browning of three primocane-fruiting raspberry cultivars. The emphasis is particularly focused on the use of suitable substance concentration to decrease explant browning and shoot necrosis during raspberry micropropagation.

MATERIALS AND METHODS

Plant materials

Primocane-fruiting raspberry cultivars 'Allgold', 'Erika', and 'Polka' were grown in the greenhouse using drip irrigation under 10-12 h photoperiod (10-12 day h) at approximately 250 $\mu moles \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) at 22-25°C with 65-75% relative humidity. Standard cultural practices including spraying fungicide were applied when needed to keep the plants healthy.

Culture initiation and subculture

Ten-centimeter long actively growing apical shoots with removal of leaves, collected from healthy plants were washed under running tap water for 15 min and then surface-sterilized in a 1% (v/v) NaOCl solution containing few drops of Tween-20 for 15 min followed by rinsing four times in sterile distilled water. Nodal segments (0.5-1 cm-long) were excised aseptically and cultured in 25 × 200 mm glass vials containing 12 mL MS medium supplemented with 30 $g \cdot L^{-1}$ sucrose, 8 $g \cdot L^{-1}$ agar, 0.6 $mg \cdot L^{-1}$ 6-benzyladenine (BA), and two kinds of supplement substances calcium gluconate and ascorbic acid. Five treatments consisting of control (0.6 $mg \cdot L^{-1}$ BA), 0.6 $mg \cdot L^{-1}$ BA + calcium gluconate (0.5 and 1 $g \cdot L^{-1}$), and 0.6 $mg \cdot L^{-1}$ BA + ascorbic acid (50 and 100 $mg \cdot L^{-1}$) were used in a completely randomized design with 10 replications (Table 1). Each replication consisted of 20 tubes and each tube contained one explant. The pH of medium was adjusted to 5.7 prior to autoclaving at 121°C for 15 min. Cultures were maintained at 23 ±2°C, recommended by Isac and Popescu (2009) for raspberry micropropagation, under a constant lighting with a PPFD of = 27 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level provided by fluorescent lamps (36 W) and subcultured every 30 days onto the same medium for shoot initiation and multiplication.

After 30 days of culture, 2-5 cm-long shoots were separated, the

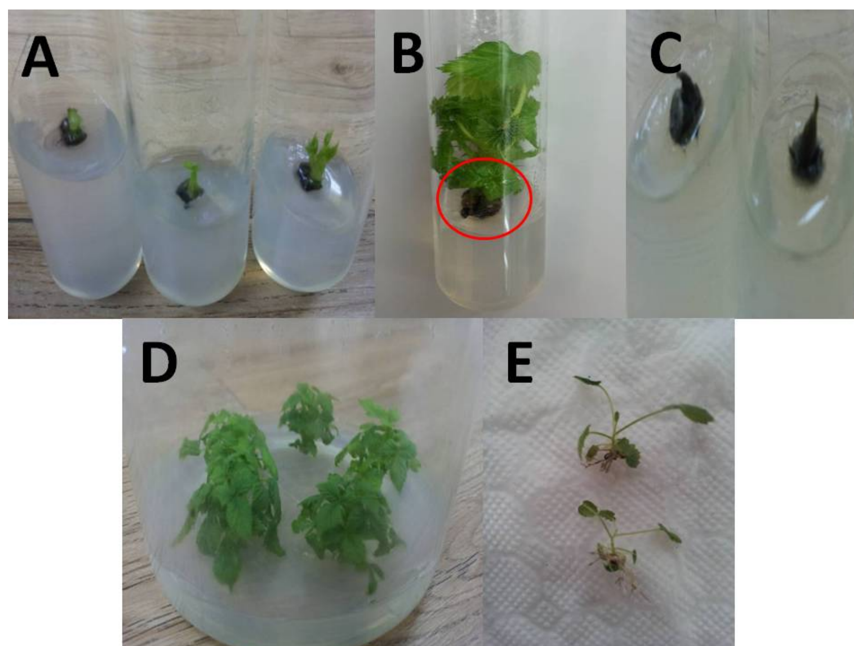

Figure 1. Primocane-fruiting raspberry micropropagation. **A,** Bud formation of 'Allgold' 'Erika' and 'Polka' respectively (from left to right), 10 days after initation of culture on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate (scale =1 : 1.25). **B.** Shoot cluster and basal callus formation within 39 days of culture initiation on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate in 'Erika' (scale =1: 1.25). C. explant browning of 'Allgold' during shoot induction stage on MS medium containing 0.6 mg·L^{-1} BA (panel; scale = 1 : 1). **D.** shoot clusters of 'Erika' at shoot multiplication stage on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate after 30 days of culture (scale =1 : 1.8). **E.** Rootted shoots of 'Allgold' after 30-45 days of culture on MS medium containing 1.0 mg·L^{-1} IBA (scale = 1 : 7).

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leaves were removed, and the stem segments (3-5 cm-long) were transferred into 330-ml light transparent polycarbonate jars containing 100 ml of similar medium used in initiation culture. The treatments then were arranged in incomplete randomized block design, block as replication, where the experimental unit was obtained from survived shoots in previous culture. The replicates were made by considering the amount of the survived shoots from previous culture in each treatment. Transferred explants developed new shoots within 30 days of culture. In subsequent subcultures during the next 30 days, the number of shoots increased substantially.

Rooting and acclimatization

Well-grown shoot clusters were seperated and cultured in 330 ml light transparent polycarbonate jars containing 100 mL of half-strength MS medium supplemented with 1 mg•L^{-1} Indole-3-butyric acid (IBA) for rooting. Each vial contained five explants and there were ten vials per treatment. Rooted shoots were removed from tissue culture after 30-45 days, rinsed free of tissue culture medium, and planted in plug trays containing peat moss + vermiculite (1:1 v/v) (Nongwoo Bio Co., Ltd., Korea). Trays were kept in a growth chamber at 23 ±2°C, covered with transparent plastic covers and gradually acclimatized to ambient humidity. After three weeks, the plants were moved into pots (14 cm-diamater, and 14 cm-deep, equivalent to 1.5 L) and placed in a greenhouse exposed to 10-12 h photoperiod (PPFD = 250 µmoles·m^{-2}·s^{-1}) at 22 - 25°C with 50-65% relative humidity. The survival rate of potted plants was recorded at one month after transfer in greenhouse.

Data collection and statistical analysis

Data were recorded for each treatment on percentage of explant browning, shoot initiation, and shoot necrosis after 30 days of culture. Observation was also done on shoot necrosis percentage, shoot number, and fresh shoot weight (g) per explant after 60 days of culture initiation. Data were subjected to analysis of variance with the SAS statistical software package (Release 9.0, SAS Institute, Inc., Cary, N.C.). Duncan's multiple range test was used to compare treatment means at $P \leq 0.05$.

RESULTS

Nodal segment of three cultivars formed buds within 10-14 days of culture. This response might be attributed to the pre-existing meristems available in the nodal segments. Shoot initiation on 'Erika' explants was observed within 10 days of culture while 'Allgold' and 'Polka' took 12-14 days for shoot initiation (Figure 1A). Analysis of variance indicated that browning explants (30 days), shoot initiation rate (30 days), and survived shoots (60 days) were different among treatments (Tables 2a and b).

Table 2a. Effects of cultivar and medium composition in raspberry micropropagation on explant browning (30 days).

Medium	Browning explant (%)		
	'Allgold'	'Erika'	'Polka'
Control	42.5 ± 0.04^{c}	27.5 ±0.08^{b}	57.5 ± 0.04^{c}
1	30.0 ± 0.02^{b}	25.0 ± 0.08^{b}	32.5 ± 0.13^{b}
2	17.5 ± 0.05^{a}	4.5 ± 0.11^{a}	17.5 ± 0.05^{a}
3	27.5 ± 0.12^{b}	37.5 ± 0.02^{bc}	35.0 ± 0.11^{b}
4	45.0 ± 0.05^{c}	62.5 ± 0.02^{d}	40.0 ± 0.15^{b}

Mean separation within columns and factors by Duncan's multiple range test; $P \leq 0.05$. Means associated with different lower-case letters signify significant differences.

Table 2b. Effects of cultivar and medium composition in raspberry micropropagation on shoot initiation rate (30 days) and survived shoots (60 days).

Medium	Shoot initiation rate (%)			Survived shoots (%)		
	'Allgold'	'Erika'	'Polka'	'Allgold'	'Erika'	'Polka'
Control	87.5 ± 0.84^{bc}	90.0 ± 0.01^{b}	70.0 ± 1.68^{b}	85.0 ± 1.19^{bc}	88.5 ± 0.65^{bc}	65.0 ± 1.19^{d}
1	90.0 ± 1.18^{b}	100.0 ± 0.04^{a}	80.0 ± 0.04^{b}	85.0 ± 1.19^{bc}	90.0 ± 1.68^{b}	65.0 ± 2.06^{d}
2	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}
3	87.5 ± 0.84^{bc}	82.5 ± 0.84^{c}	90.0 ± 0.04^{b}	77.5 ± 0.84^{cd}	62.5 ± 1.88^{d}	72.5 ± 1.88^{cd}
4	82.5 ± 0.84^{c}	70.0 ± 1.19^{d}	80.0 ± 1.68^{b}	62.5 ± 0.84^{d}	72.5 ± 1.46^{d}	77.5 ± 1.19^{cd}

Mean separation within columns and factors by Duncan's multiple range test; $P \leq 0.05$. Means associated with different lower-case letters signify significant differences.

An addition of 1 mg·L^{-1} calcium gluconate into MS medium containing 0.6 mg·L^{-1} BA at shoot induction stage resulted in highest shoot development rate for three cultivars. Cultivar 'Polka' showed the lowest survival rate (observed at 60 days after initiation). Clusters of 2-3 shoots per explant were developed within 30 days in all three cultivars. Nodal segments also developed calli at the base of each explant (Figure 1B). Browning symptoms appeared at first seven days of culture initiation. Severe browning symptoms on the entire parts of explants caused the death of explants that makes them unable to generate shoot (Figure 1C).

After 30 days of culture initiation, the initiated shoots were sub-cultured into the same medium for shoot multiplication. The shoot necrosis symptoms appeared at this stage. Addition of 1 g·L^{-1} calcium gluconate was observed to be effective in reducing frequency of shoot necrosis (Figure 2). Despite its beneficial effect to reduce shoot necrosis symptoms on *in vitro* culture, addition of calcium gluconate resulted in slower growth of shoot and reduced multiplication rate for cultivars, 'Allgold' and 'Erika', compared to control medium (BA 0.6 mg·L^{-1}). However, in 'Polka', addition of 1 g·L^{-1} calcium gluconate into culture medium reduced shoot necrosis symptoms, but did not reduce the rate of shoot growth. Presence of ascorbic acid at both concentration of 50 and 100 mg·L^{-1} in culture medium slightly prevented necrosis in 'Polka' and 'Allgold, but not in 'Erika'.

Browning symptoms and shoot necrosis showed negative correlation with shoot initiation rate and survived shoots. The lower explants browning and shoot necrosis resulted in higher shoot initiation rate and survived shoots and vice versa (Figure 3). There were significant differences on shoot number and shoot weight among cultivars and medium compositions after 60 days of culture (Figure 4A). Slow shoot multiplication of 'Allgold' and 'Erika' due to addition of calcium gluconate was indicated by their lower number of shoots per explant than the control (Figure 4A). Calcium gluconate supple-mentation also led to lower weight of shoot on cultivar 'Erika' (Figure 4B). On the other hand, addition of ascorbic acid into culture medium significantly increased shoot weight of all cultivars. The shoot weight of 'Polka' was increased with increasing the concentrantion of ascorbic acid. Shoot weight of 'Allgold' and 'Erika' increased at 50 mg·L^{-1} ascorbic acid but decreased at a concentration of 100 mg·L^{-1} (Figure 4B).

After 30-45 days of culture in shoot multiplication medium, raspberry shoot clusters were ready to transfer onto the rooting medium. Culture shoots rooted within 30-45 days (Figure 1E) and they were planted and main-tained in the greenhouse. The survival rate of acclimated planlets reached 90-95% and abnormalities in plant development were not observed.

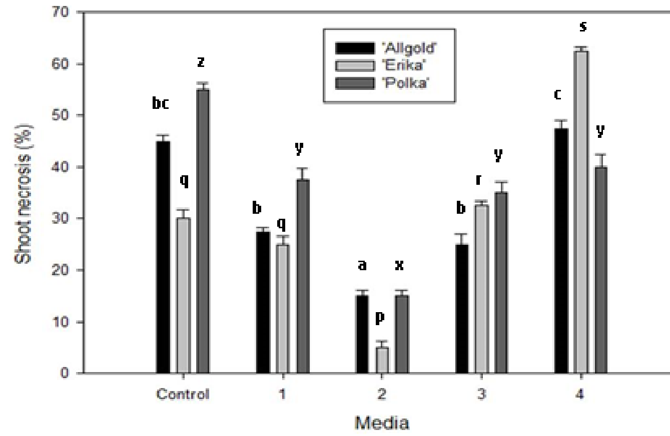

Figure 2. Effects of calcium gluconate and ascorbic acid on shoot necrosis after 60 days of culture on different culture medium compositions. Bars associated with different low case letter show significant difference within cultivar.

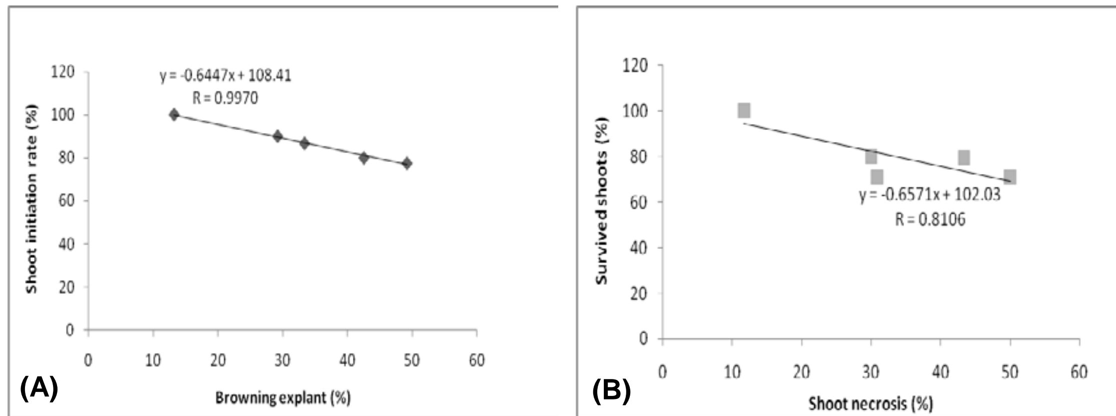

Figure 3. Negative correlation of browning explants on shoot initiation rate (A) and shoot necrosis on survived shoots (B).

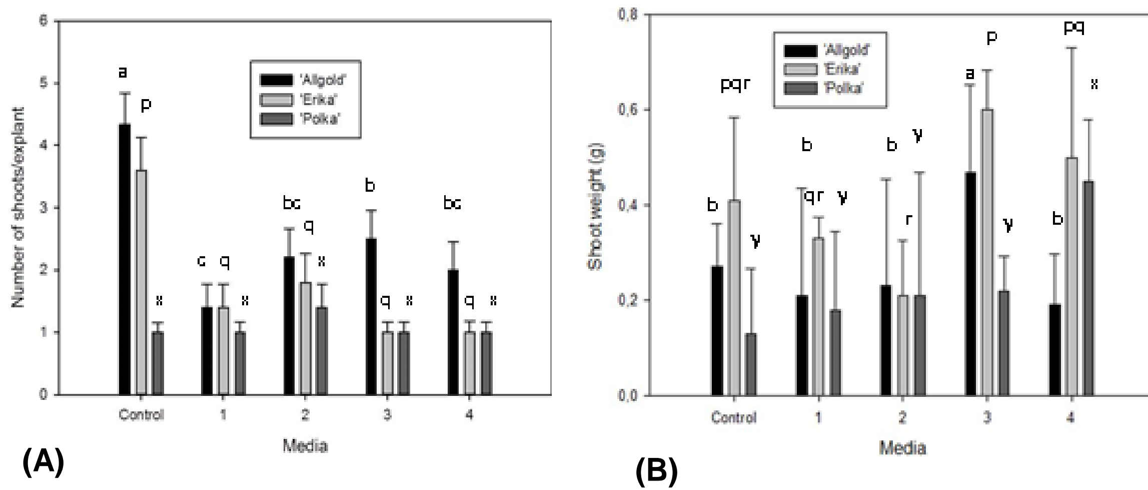

Figure 4. Effects of calcium gluconate and ascorbic acid on number of shoots/explant (A) and shoot weight (B) after 60 days of culture on different culture medium compositions. Bars associated with different low case letter show significant difference within cultivar.

DISCUSSION

Effects of genotype on shoot initiation rate

Raspberry cultivars used in this study required different period to initiate shoot. Node culture of 'Erika' showed shoot initiation earlier than those of 'Polka' and 'Allgold'. Plant genotype often profoundly affects explant response to *in vitro* culture as proposed by Burbulis et al. (2012). Similar observation on other raspberry cultivars was reported by Georgieva et al. (2004) where raspberry cultivar 'Bulgarski Rubin' started shoot initiation three weeks after culture initiation while 'Shopska Alena' started one week later. The different responses of raspberry cultivars in *in vitro* culture might be caused by the endogenous hormonal balance in plant tissue as mentioned by Tanaka et al. (2012) that the difference in adventitious bud regeneration between cultivars might be caused by differences in endogeneous hormones levels between cultivars.

Effects of calcium gluconate on shoot necrosis and plant growth

To eliminate shoot necrosis during micropropagation, calcium gluconate was used in a range recommended by previous reports on same cases. The use of 0.5 and 1 $g \cdot L^{-1}$ calcium gluconate in this experiment was in consideration with the finding of Singha et al. (1990) which mentioned that 0.03-3.0 $g \cdot L^{-1}$ calcium gluconate could effectively reduce shoot tip necrosis in shoot cultures of quince. Visible symptoms of shoot necrosis in primocane-fruiting raspberry culture showed a pale brown necrosis developed at the tips and margins of young leaves that spread to whole microshoots, and subsequently became darkened and eventually, the shoots died. These symptoms also occurred in *Pistachia* meristem culture where calcium deficiency was a problem (Abousalim and Mantell, 1994). Calcium is a major anion in plant cell and plays a key role in many physiological processes in plants. Thus, calcium deficiency could result in disturbance of metabolic activities of growing tissues, which in turn result in growth abnormalities such as shoot necrosis (Bairu et al., 2008).

Addition of 1 $g \cdot L^{-1}$ calcium gluconate was found to be effective to reduce frequency of explants browning and shoot necrosis on all raspberry cultivars. Alleviation of shoot necrosis by using calcium gluconate to increase calcium concentration in culture medium, as in this study, has been demonstrated in Pistachio (*Pistacia vera*) (Abousalim and Mantell, 1994), where calcium supplementation in MS medium using 1.5 and 3.0 $g \cdot L^{-1}$ calcium gluconate significantly decreased shoot tip necrosis and increased shoot length and bud number. Despite its benefit to reduce shoot necrosis, presence of calcium gluconate in medium reduced shoot multiplication rate of cultivars 'Allgold' and 'Erika'. An increase of calcium salts in medium of quince culture was reported to cause reduction of shoot proliferation and growth (Singha et al., 1990). Giel and Bojarczuk (2011) mentioned that small amount of calcium salts in the growth substrate stimulates seedling growth of rhododendron cutting, while an excessively high calcium content of the substrate, inhibited their growth and development.

The reduction of raspberry explant growth in cultivar 'Allgold' and 'Erika' is suggested as an indirect influence of calcium on plant cell as proposed by Singha et al. (1990). One of the important roles of calcium within plant cells is to determine the structural rigidity of the cell wall. High concentration of calcium will rigidify the cell wall and make it less plastic resulting in slow cell division and tissue elongation (Hepler, 2005). Thus, from this mechanism, it can be assumed that elevating the Ca^{2+} concentration could reduce shoot multiplication. However, the effects of calcium on growth and development of plants is a complicated mechanism as Ca^{2+} influences plant growth through its role in cell wall structure, cellular membrane system, cell division, and its interaction with plant growth regulators (Poovaiah and Leopold, 1973) .

In this study, differential expression of shoot multiplication rate among genotypes of primocane-fruiting raspberry cultivars was observed in the presence of calcium gluconate in culture medium. These results could be attributed to the complicated interactions between calcium and plant hormones, which is varied among cultivars. Santner et al. (2009) suggested that many growth regulators (plant hormones) could potentially affect the plant susceptibility to Ca^{2+}. Specific growth regulator produced at different plant tissues, cultivars and developmental stages could potentially regulate plant Ca^{2+} uptake and translocation.

Effects of ascorbic acid on shoot necrosis and plant growth

To eliminate shoot necrosis during micropropagation, ascorbic acid in a dosage of 50 and 100 $mg \bullet L^{-1}$ was also used in this experiment by considering recommendation by Cassels and Minas (1983). They suggested that the high concentration of ascorbic acid (above 50 $mg \bullet L^{-1}$) is effective to control the shoot necrosis on *Pelargonium* micropropagation.

Ko et al. (2009) also mentioned that the use of ascorbic acid was found to be effective to prevent the development of lethal browning on tissue culture of cavendish banana. In this study, addition of ascorbic acid in culture medium significantly reduced explant browning and shoot necrosis on cultivar 'Polka' although less effectively than the addition of calcium gluconate. On the other hand, addition of 100 $mg \cdot L^{-1}$ ascorbic acid resulted in higher explant browning and shoot necrosis on cultivar 'Erika' compared to control medium. Different response of

raspberry cultivars to application of ascorbic acid can be resulted from the interaction between ascorbic acid and phenolic compounds. Ascorbic acid in high doses causes the production of large amount of hydroxyl radicals by reducing oxygen in the presence of transition iron and copper (John and Borut, 2007) available in culture medium. The large amount of hydroxyl radicals was absorbed and quenched with the excess of phenols (Jin and Russel, 2010) produced by cultivar 'Polka' while in cultivar 'Erika', the production of phenols might be not sufficient to extinguish those hydroxyl radicals. As the level of hydroxyl radicals overcome endogenous phenolic content in 'Erika', oxidative reactions might intensively occur and lead to high level of explant browning and necrosis (Jin and Russel, 2010).

Presence of ascorbic acid in culture medium of three cultivars of primocane-fruiting raspberry significantly affected the growth of cultures. The response of each cultivar on the addition of ascorbic acid was different. In cultivar 'Polka', shoot weight increased with the increase of ascorbic acid concentration to 100 mg·L^{-1}, while in 'Allgold' and 'Erika', it increased at 50 mg·L^{-1} ascorbic acid and then decreased when the concentration was 100 mg·L^{-1}. The decreased weight could be due to high level of explants browning and necrosis in 'Allgold' and 'Erika' at 100 mg·L^{-1} ascorbic acid. The effects of ascorbic acid on the plant growth were previously reported in some crops. Bathia and Ashwath (2008) found that addition of ascorbic acid in the range of 30-480 µM into medium could significantly promote *in vitro* shoot growth of tomato. They stated that quality of regenerated shoots could be improved by using ascorbic acid due to its function to oxidize growth inhibitory substances produced in tomato *in vitro* culture. Antioxidants including ascorbic acid may affect morphogenic processes by providing protection against oxidative stress (Leshem, 1988). Addition of antioxidants into tissue culture medium also enhances the development of isolated cells and tissues (Khan et al., 2011).

In conclusion, the present study clearly documented the effects of calcium gluconate and ascorbic acid on the shoot necrosis and growth of explants *in vitro* from nodal segments. Diverse responses were observed for shoot initiation rate among primocane-fruiting raspberry cultivars related to calcium deficiency that induced necrosis and dieback of explants. Generally, addition of calcium gluconate decreased explants browning and shoot necrosis during micropropagation of all raspberry cultivars used in this experiment. The decreasing explants browning and shoot necrosis then resulted in increasing shoot initiation rate and survived shoots. Therefore, an addition of 1 g·L^{-1} calcium gluconate into MS medium containing 0.6 mg·L^{-1} BA at shoot induction stage is recommended to promote shoot initiation and to get more viable shoots. We suggest this method is sui-table for shoot multiplication without explant browning and shoot necrosis.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

The influence of soil properties on the development of *Cephalcia tannourinensis* Chevin (Hym. Pamphiliidae) infesting the cedar forests in Lebanon

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The cedar web-spinning sawfly, *Cephalcia tannourinensis* (Hymenoptera: Pamphiliidae) is considered a major defoliator of the cedar forests in Lebanon. In the early 1990's, a severe damage of the Tannourine Hadath El-Jebbeh cedar forest was caused by the sudden outbreak of this insect. The insect has a complex life cycle with two types of diapause: annual diapause and multiple years diapause. The present study aimed at studying soil characteristics and their influence on the survival and diapausing cycles of *C. tannourinensis* prepupae. Different soil samples collected from three different cedars forests in Lebanon were incubated with prepupae of *C. tannourinensis* under laboratory conditions. The effect of soil origins and properties on prepupal mortality, annual diapause and multiple years diapause cycles were analyzed. The influence of soil moisture and soil temperature on prepupal development was studied separately through two constant temperatures and three constant soil moisture levels. Prepupal mortality was found to be highly correlated with soil properties and types. Prepupae survived well in soil coming from Tannourine and Bcharry followed by Chouf. However mortality was almost 100% in the two reference soils sand and peat. Diapause cycle was strongly correlated with temperature while the effect of moisture was mainly on the prepupal survival.

Key words: *Cephalcia tannourinensis*, soil moisture, soil temperature, annual diapause, multiple years diapauses.

INTRODUCTION

Cedrus libani A. Richard (Pinaceae) is a majestic tree of 15 to 40 m height at maturity with innumerable scriptural and historical references. It is by far the most famous and treasured tree in Lebanon. Historically, it was mentioned in mythology and cited 75 times in the Holy Bible and is used as symbol of strength and stability. References to

the Cedars of Lebanon go as far back as the beginning of written script and the first temple of Solomon was built with it. In earlier times, the Cedar of Lebanon covered a large area of Lebanon's mountains. Thus, the cedar became Lebanon's national emblem and it is seen on the Lebanese flag.

In Lebanon, *C. libani* is an important forest tree species between 1000 – 2300 m and most abundant on North-facing slopes, where the impact of radiation is less severe, but in wetter locations it grows equally well on the mountain sides exposed to the prevailing rain-bringing winds (Khuri et al., 2000). Twelve (12) separate regions are known to harbor the Cedar in Lebanon: Jabal Qammoua forest, Wadi Jahannam in the Akkar area, Ehden, Bcharre, Tannourine-Hadeth, Jeij in the Jbail Mountains, and in the Jabal el-Barouk forests of the Chouf mountains, Ain Zhalta/Bmohrain, Barouk, and Maasser el-Chouf.

Cephalcia tannourinensis (Hymenoptera: Pamphiliidae), the cedar web-spinning sawfly was described as a new species by Chevin (2002). Between 1990 and 1999, the intensity had been increasing in Tannourine-Hadath El-Jebbeh cedar forest located in northern Lebanon and has caused intense defoliation of over 600 ha.

The larvae feed on cedar needles and after the last molt, they drop from the crown to the ground where they hibernate (Nemer et al., 2005). The larval diapause can last for more than one year; and a five year diapause was recorded for some species within the same genus (Gruppe, 1996). Aerial spraying with the insect growth regulator diflubenzuron was carried out in 1999-2004 resulting in considerable suppression of the cedar web-spinning sawfly population (Nemer and Nasr, 2004) but for ecological reasons development of more environmentally friendly techniques to manage populations are required (Nemer et al., 2007; Abdo et al., 2008). The danger of the spread of this insect to other cedar forests in Lebanon and the Mediterranean region led to the initiation of a UNEP/GEF project entitled "Integrated Management of Cedar Forests in Lebanon in Cooperation with Other Mediterranean Countries" that aims to develop an action plan for integrated management of forests including assessment of insects in cedar forest in the Mediterranean region with particular emphasis on the Tannourine-Hadath El-Jebbeh cedar forest. The causes of *Cephalcia* outbreak in the forest of Tannourine-Hadath El-Jebbeh are still not known. A variety of factors may have contributed to the population increase of *C. tannourinensis* during the last decade. Climatic factors and other characteristics related to the microhabitat of the forest ecosystem are thought to be among the principle causes of this outbreak. Subsequently, it was decided to initiate the present study with the following objectives: (i) determine the relationship between soil properties and survival of the prepupae of *C. tannourinensis* larvae as well as the annual and multi annual diapause development of this insect and (ii) understand the relation between soil temperatures and soil moisture vis-à-vis the development of the prepupae of *C. tannourinensis.*

MATERIALS AND METHODS

Study site

The field studies were conducted in the Tannourine cedar forest. The cedar forest of Tannourine Hadath El-Jebbeh is located in North Lebanon about 85 Km from Beirut. It falls between 34º12' and 34°15' of Latitude N and between 35°54' and 35º56' of Longitude W from Greenwich. The altitude varies between 1,430 to 1,850 m above sea level. The forest is physiologically positioned on a moderately steep sloping valley composed mostly of alluvial deposits of calcareous and basaltic deposits. The forest area is located in the supramediterranean region with an annual rainfall varying between 1,200 to 1,400 mm Food and Agriculture Organization, 2003).

Laboratory studies

Effect of different soils on the development of prepupae of C. tannourinensis

Soil sampling and analysis: Collection of soil samples and methods of analysis were done according to Bashour and Sayegh (2001). Composite soil samples were collected from Tannourine cedar forest, Maasser El-Chouf, Hadath El-Jebbeh and Bcharry (Table 1) during the first week of July 2007, which coincided to the period where mature larvae of *C. tannourinensis* dropping to the ground and penetrating the soil. Approximately 2 kg of soil were taken from each sampling site at a depth of 0 to 15 cm using a hand pulled auger. Soil clods were broken down and the content was mixed well. The composite sample was transferred to a clean plastic bag after being labeled. The bags were closed tightly and transported to the laboratory where each sample of soil was spread to air dry on a clean paper sheet at room temperature. Soil aggregates were also broken down by hand using gloves to accelerate the drying process. After 10 days each sample was crushed and ground to particles of uniform size using porcelain crushers and sieved through a 2-mm sieve. Then each sample was stored properly in a well labeled and air tight clean plastic container for further analysis.

(i) Moisture content: Before air drying the samples, an amount of approximately 200 g of fresh soil was taken from each bag and transferred to a weighed metal can with a tight fitting lid; the weight of the can and the soil was taken using a mettler balance. The cans

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Table 1. Geographic positions of the different soils samples.

Soil	Location	Northing	Easting	Altitude (m)
T1	Tannourine, Jourit El-Akhrass	34° 12'.517	35° 55'.699	1.768
T2	Tannourine Ain El Raha	34° 12'.485	35° 55'.510	1.733
M3	Maasser El-Chouf	33° 40'.635	35° 41'.578	1.735
M4	Maasser El-Chouf	33° 40' .672	35° 41'.489	1.710
H1	Hadath El-Jebbeh	34° 13'.005	35° 56'.001	1.756
H2	Hadath El-Jebbeh	34° 13'.001	35° 56'.007	1.692
B1	Bcharry	34° 14'.571	36° 02'.905	1.901

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

were placed in a drying oven at 110°C for a period of 24 h, cooled in a dessicator and weighed again. Moisture content was calculated according to the following formula:

Weight of water = Weight of air dried soil – Weight of oven dried soil

% Moisture content = (Weight of Water / Weight of oven dried soil) x 100

(ii) Organic matter content (combustion method): The oven dried soil samples described above were placed in a furnace at 350°C for four hours. The cans were then closed tightly and transferred into desiccators in order to cool; then were weighed again. Organic matter content was calculated on oven dried basis (Bashour and Sayegh, 2001).

(iii) Soil texture: The soil particle size distribution was determined by the Bouyoucos Hydrometer Method. Fifty grams of oven dried fine texture soil were taken from each sample and then transferred into a baffled stirring cup; and was filled with distilled water to half full, 15 ml of sodium hexametaphosphate (1N) was also added. Then the mixture was stirred in an electrically driven mixer for 10 min. The stirred mixture was transferred to a settling cylinder, the soil suspension was shaken vigorously in a back and forth manner. After 20 s, the hydrometer was inserted into the cylinder and the readings were taken after 40 s. This reading indicated the percentage of clay and silt particles in the soil suspension. A second reading was taken after 2 h for the determination of clay. Temperature was measured and corrections were done according to the outlined procedure in (Bashour and Sayegh, 2001).

(iv) Exchangeable cations: The exchangeable calcium (Ca), sodium (Na), potassium (K) and magnesium (Mg) contents were measured by taking 3 g of air dried soil from each sample and transferring them into a plastic falcon tube. A volume of 30 ml 1.0 N ammonium acetate were added to each sample and the mixture was shaken on an orbital shaker for 30 min, then all the samples were filtered and the filtrate were stored in a refrigerator at 4°C for further analysis. Ca, Na and K were measured using an AFP 100 Flame photometer, (Biotech Engineering management Co. LTD, U.S.A.) and Mg was measured using an AA 6300 Atomic absorbance spectrophotometer (SHIMADZU, Japan) (Bashour and Sayegh, 2001).

(v) Micronutrients (Fe, Zn, Mn and Cu) analysis: Available micronutrients in soil (iron, zinc, manganese and copper) were extracted by taking about 5 g of air dried soil were taken from each sample and transferred to a plastic falcon tube. A volume of 20 ml of diethylenetriaminepentaacetic acid (DTPA) solution was added to each tube. The tubes were then shaken on an orbital shaker for a period of 30 min. and filtered using a Whatman filter paper No. 40 in order to be measured on an AA 6300 Atomic absorbance spectrophotometer (SHIMADZU, Japan) (Bashour and Sayegh, 2001).

(vi) Available phosphorus (P): The determination of available phosphorus in the soils was done by placing 3 g of soil from each air dried soil sample into a 100 ml falcon tubes. 50 ml of sodium bicarbonate (0.5 M) were added to each tube and placed on the shaker for 30 min. The mixtures were then filtered using a Whatman filter paper No. 40 and a clear solution was attained. Eight drops of Sulfuric acid (H_2SO_4 2.5 M) were added to lower soil pH. Molybdate solution and 8 ml ascorbic acid were added, swirled and washed with distilled water in order to remove the $C0_2$ gas and form a complex blue color. Phosphorus was measured on an Aquamate spectrophotometer (Thermo Electron Corporation, USA) (Bashour and Sayegh, 2001).

(vii) Free Calcium Carbonate ($CaCO_3$) Content: 3 g from soil sample were placed in 250 ml beaker, 30 ml of standardized (1.0 N) hydrochloric acid (HCL) were added. The beakers were each covered by a watch glass and boiled for 5 min then cooled down and filtered using a Whatman filter paper No. 40. Phenolphthalein indicator was added to the excess acid. The filtrates were measured by back titration with 0.5 N sodium hydroxide (NaOH) (Bashour and Sayegh, 2001).

(viii) pH and electric conductivity (EC): A total weight of 10 g of each air dried soil sample were transferred into a 50 ml falcon tube, 25 ml of distilled water were added to each tube and then shaken on an electric shaker for 30 min. The mixtures were then filtered using a Whatman filter paper No. 41 and the pH values were measured by a Thermo Orion pH meter model 410 A +. For comparison to a peat moss sample (Russian made), a saturation paste was made of 10 g. of peat and 70 ml of distilled water in a covered beaker for a period of 24 h. Afterwards, the paste was filtered on a Whatman filter paper No. 41 and the filtrate was transferred to a clean 200 ml falcon tube. EC was measured using a Mettler Toledo, M226 Conductivity meter. The readings were preformed under constant laboratory temperature at 24°C (Bashour and Sayegh, 2001).

(ix) Cation exchange capacity (CEC): About 1 g were taken from each soil sample and transferred to a 200 ml falcon tube to which a volume of 8 ml 1 N Sodium acetate (NaOAC) was added. The tubes were shaken for 5 min on an electric shaker. Then the caps and the sides of the tubes were washed well with NaOAC solution. The clean tubes were centrifuged for 3 min. at a speed of 2000 rpm in a

Durafuge 200 centrifuge (Precision, France). The supernatant was discarded. This operation was repeated three times. Afterwards, the samples were washed four times with 8 ml ethanol and centrifuged. The EC of the supernatant became 40 microseimens per cm. or even less. Then 8 ml of ammonium acetate (NH_4OAC) were added to each sample and centrifuged for 3 min, the supernatants were collected and placed into a 50 ml volumetric flask. Sodium (Na) was measured directly on an AFP 100 Flame photometer (Biotech Engineering Management Co. LTD, USA) (Bashour and Sayegh, 2001).

Experimental setup

Cedar branches infested with second instar larvae of *C. tannourinensis* were collected from Tannourine forest during the month of June 2007 and brought to the laboratory where they were placed in an incubator set at 20°C with a photoperiod of 14:10 (L:D). The larvae were allowed to mature under these conditions. Prepupae (mature larvae) were collected once they ceased feeding and dropped from cedar branches. The prepupae were then transferred to the different soil types (Table 1) in addition to two reference soil types namely peat moss (Kitexim, Kaliningrad, Russia) and pure sand. A total weight of 40 g of soil was taken from each soil type with the exception of peat moss where only 20 g were taken. The soils were placed in 50 ml glass beakers. The prepupae were placed on the top of soil and allowed to penetrate freely in it. The beakers were then sealed with parafilm in order to reduce evaporation and thus maintain a relatively constant humidity. A total number of six prepupae were placed in each beaker and each soil type was replicated three times. The total number of soil types and origin was nine. The beakers were placed in the incubator under a constant temperature of 20°C equal to the average soil temperature observed in the forest of Tannourine during the months of June and July.

Data on prepupal mortality, number of prepupae that developed into pronymphs (complete eye development) and those into eonymphs (absence of eye formation) were taken after 105 days. This period is necessary for complete eye formation based on field observations at the Tannourine cedar forest.

Statistical analysis

The experiment was a completely randomized design with one factor, the soil origin and types. ANOVA 1 was performed and means were separated by LSD. Correlation and linear regression analysis were performed between the different soil factors (moisture, nutrients, texture, etc.) and the larval mortality and the formation of eye.

Effect of soil temperature on the development of Prepupae of *C. tannourinensis*

Soil sampling

In this experiment, soils from Tannourine forest only were used since prepupae of *C. tannourinensis* are known to live in these soils. Soil samples from a previously outbreak area in the Tannourine forest were collected in sealed plastic bags in July 2007 and directly used in the experiment to reduce humidity loss.

Experimental setup

The experiment was set as described previously in (soil sampling and analysis/experimental setup) and each beaker was sealed with parafilm after the prepupae were placed on top of the soil. Treatments were replicated three times and 20 prepupae were used per treatment. Three replicates of each treatment were placed in an incubator held at 20°C and another three replicates were placed in an incubator held at 10°C. Data on larval mortality, number of prepupae that developed into pronymphs (complete eye development) and those into eonymphs (absence of eye formation) were taken after 105 days.

Statistical analysis

The number of dead prepupae, pronymphs and eonymphs were analyzed by t-test by comparing the means of both temperature regimes.

Effect of soil moisture on the development of Prepupae of *C. tannourinensis*

Soil sampling

Soil samples from a previous outbreak area in the Tannourine forest were collected in July 2007. Soil moisture was adjusted to three levels: 5, 20 and 40%. The soil moisture of the samples was determined following the method described in experiment (soil sampling and analysis). The adjustments of moisture were done by either adding distilled water to the sample to reach the level required or oven drying the samples and regulate the measurement to reach the required moisture level.

Experimental setup

The experiment was set as described in experiment (effect of soil temperature on the development of Prepupae of *C. tannourinensis* / soil sampling) and each beaker was sealed with parafilm after the prepupae were placed on top of the soil. Soil samples were replicated three times with 10 prepupae in each for each moisture level: 5, 20 and 40%. The sealed beakers were placed in an incubator held at 20°C. Data on larval mortality, number of larvae that developed into pronymphs (complete eye development) and those into eonymphs (absence of eye formation) were taken after 105 days.

Statistical analysis

The design of the experiment was set as a completely randomized design (CRD) with one factor, soil moisture. ANOVA 1 was performed and means were separated by LSD. Linear and nonlinear curves were determined to evaluate the relationship between moisture levels and eonymphs.

RESULTS AND DISCUSSION

Effect of different soils on the development of Prepupae of *C. tannourinensis*

The main purpose of this experiment was to study the effect of different soil physical and chemical properties and their influence on the development of the *C. tannourinensis* prepupae conducted under stable laboratory conditions (temperature, light and moisture).

Table 2. Moisture levels and organic matter content of soil samples (depth 15-30 cm) collected from different cedar forests in Lebanon on June 16, 2007.

Soil sample	Moisture content (%)	Organic matter content (%)
T1	20.9	17.0
T2	17.0	11.4
M3	5.7	7.5
M4	18.9	10.8
H1	14.5	6.4
H2	14.0	10.1
B1	12.3	12.2
Sand	0.5	0.0

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

Table 3. Textural class of the different soil samples (Depth 15-30 cm) collected from different cedar forests in Lebanon on June 16, 2007.

Soil sample	Sand %	Silt %	Clay %	Textural class
T1	26	28	46	Clay
T2	22	28	50	Clay
M3	34	36	30	Clay loam
M4	12	24	64	Clay
H1	32	24	44	Clay
H2	26	28	46	Clay
B1	28	40	32	Clay loam

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

Soil origins and analysis

Moisture content

Soil samples were collected within two days during the second week of June 2007. Soil moisture measurements showed an elevated moisture percentage in the samples from Tannourine forest (20.9 and 17.0%) followed by one location in Maasser El-Chouf (M4) (18.9%). The moisture percentage in H1 and H2 from Hadath El-Jebbeh forest were 14 and 14.5 %, respectively (Table 3). Soil moisture content depended on the season and the annual precipitation of a specific region.

Organic matter content

Soil organic matter varies between 1 and 5% in most Lebanese arid and semi arid regions. This was not the case in our soil samples due to the fact that the samples were taken from cedar forests. The dense canopy of these forests affected the soil organic matter and raises the percentage to levels ranging between 6.4 and 17% (Table 2).

Soil texture

The results of soil particle size distribution showed that the soil samples from different locations are of clayey type and can be further classified into Red Mediterranean soil (Table 3). The clayey Mediterranean soil is characterized by complex and diverse parental material with various drainage conditions and seasonal moisture fluctuation. The soil samples can be further classified into Reddish Brown and Red Ferrallitic soils and they were first described in 1930 in many Mediterranean countries under the name of Terra-Rossa (Marcelin, 1942; Bashour and Sayegh, 2001).

Exchangeable cations (Ca, Na, K, and Mg) analysis

The results for exchangeable cations are presented in

Table 4. Levels of soil exchangeable cations (K, Ca, Na, and Mg) in the soil samples taken from different cedar forests in Lebanon at a depth of 15 to 30 cm.

Soil sample	K (mg/kg)	Ca (mg/kg)	Na (mg/kg)	Mg (mg/kg)
T1	550	4825	155	310
T2	530	8400	113	381
M3	540	9120	145	352
M4	57	7470	163	453
H1	400	7800	136	520
H2	480	7250	113	305
B1	530	7000	107	512

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

Table 5. Micronutrients (Fe, Zn, Mn and Cu) levels and soil phosphorous ($NaHCO_3$–P) values and Calcium Carbonate percentages in the soil samples taken in June 2007 from different cedar forests in Lebanon at a depth of 15 to 30 cm.

Soil sample	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Phosphorous (mg/kg)	Calcium arbonate ($CaCO_3$)%
T1	162.0	3.4	186.1	2.1	13	0.6
T2	30.0	2.3	59.9	1.9	10	0.9
M3	20.0	1.5	10.9	1.2	20	13.5
M4	30.0	2.0	22.3	1.8	29	1.3
H1	17.0	0.9	24.7	2.2	10	0.1
H2	20.0	1.1	33.4	2.3	7	0.1
B1	16.0	1.4	9.0	0.9	15	12.7

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

table 4. Results indicate that the Tannourine soil is very rich in potassium (K); because available K which was extracted with NH_4OAC was higher than 500 mg/kg. This is due to the mineral types and texture of the soil which is clayey. Clayey soils prohibit the fast leaching of K which originates from the fresh plant and tree residues. K is very leachable in humus or well decomposed plant material. Similar levels of K were obtained in Hadath-El-Jebbeh, Bcharry and one location of Maasser El-Chouf, where K was higher than 300 mg/kg, except for one sample M4 from the Maasser El-Chouf cedar forest. The low K level of M4 sample may be due to the high rainfall, snow rates and high leaching in addition to the lack of vegetation cover to protect soil from strong wind which prevails in the area which blows away the fine mineral soil particles.

Calcium (Ca) levels were higher than 3500 mg/kg in all soil samples (Table 4); this is considered very high according to Bashour and Sayegh (2001). The levels of sodium (Na) in all the samples was medium/low (Table 4) according to Bashour and Sayegh (2001). Soil Na ranged between 100 and 150 mg/kg. Unlike other areas in Lebanon, the Tannourine cedar forest samples belong to a wild uncultivated soil having undisturbed profiles with good infiltration levels, therefore salinity levels are way below the damaging threshold and salt content in soils do not cause problems for plant growth. Results for magnesium (Mg) indicated that its level in the soil samples varied between high to very high (300 and 500 mg/kg) Bashour and Sayegh (2001) (Table 4).

Micronutrients (Fe, Zn, Mn and Cu) analysis

The soil micronutrients are essential for plant growth and are needed in minute quantities. They originate from weathered minerals, are more soluble in acidic solutions or acidic soils, are directly influenced by high pH, and they are usually low in soils of arid regions especially calcareous soils.

Results showed that the soil samples were very high in iron (>10mg/kg) Bashour and Sayegh (2001) (Table 5). This may be due to the nature of parent rocks and the high levels of humus and organic matter in the soil. Also the results of available Mn, and Cu were high in the soil. As for the available Zinc (Zn), its level was found to be medium in all the samples, ranging between 1.2 to 4 mg/kg.

Table 6. Soil pH, electrical conductivity (EC) and cation exchange capacity (CEC) values soil samples collected from different cedar forests in Lebanon.

Soil sample	pH	EC (µS/cm)	CEC (meq/100g)
T1	8.1	539.0	47.4
T2	7.9	224.0	55.2
M3	8.1	138.6	51.3
M4	8.1	176.9	50.0
H1	7.8	105.4	51.5
H2	8.0	166.7	47.2
B1	8.1	138.7	41.0

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

Available phosphorus (P)

Results of available phosphorus revealed that Maasser El-Chouf soils have the highest levels followed by Bcharry with 15 mg/kg, then Tannourine with 13 and 10 mg/kg in both samples and finally by Hadath El-Jebbeh (7-10 mg/kg) the least amount of available phosphorous which is considered sufficient for forest growth (Table 5).

Free calcium carbonate ($CaCO_3$) content

Results of calcium carbonate analysis (Table 5) indicated that Tannourine soils are very low in $CaCO_3$ according to the recommended ranges Bashour and Sayegh (2001). Results of analysis indicated that samples (M3 and M4) collected from Maasser El-Chouf were high in $CaCO_3$ levels. The other five samples collected from Bcharry and Hadeth El-Jebbeh forests were medium in $CaCO_3$ content. Calcium carbonate affects the moisture characteristics of soils. It can help in the formation of soil aggregates and interferes in its particle size distribution. Moreover, $CaCO_3$ can be considered as a soil cementing agent helping or improving the formation of soil aggregates.

pH and electric conductivity (EC)

Soil pH of Lebanese cedar forests was found to be slightly basic with minute differences between the different forests (Table 6). Electrical conductivity (EC) is used to determine the total concentration of solutes and therefore reflects the salinity levels of soil; it has a critical effect on the germination and emergence stages of seedlings. Results showed that soils of different cedar forests have low EC as it is the case in all Lebanese soils.

Cation exchange capacity (CEC)

CEC is defined as the number of cation adsorption sites per unit weight of soil or the total sum of exchangeable cations that a soil can adsorb. CEC is expressed in centimole per kilogram (cmole/kg) of oven dried soil. CEC values were found to be high in all forest soil samples with minor differences between them (Table 6). A high CEC value (>25 meq/100 g) is a good indicator for a soil rich in clay and organic matter content, therefore it will hold high amounts of cations and reduce leaching Bashour and Sayegh (2001).

Laboratory experiment with Prepupae of *Cephalcia tannourinensis*

Prepupa mortality was significantly affected by soil types (ANOVA; $F = 10.827$; $df = 8, 26$; $P < 0.0001$) (Table 7). *C. tannourinensis* prepupa survived well in soils coming from Tannourine (T1 and T2), where the maximum mortality was 27.8%. Prepupae have constructed an earth cell chamber and thus protected themselves from physical and biological disturbances. The highest mortality was observed in soils originating from Maasser El-Chouf (M3) and in the two reference soils, namely peat and sand where the mortalities were 100, 77.8 and 100%, respectively. The Hadath El- Jebbeh soil (H2), the Maasser El-Chouf (M4) and the soil of Bcharry (B1) showed similar mortality rate to that of the two samples from Tannourine (T1 and T2).

Observations showed that the high mortality in H1 was related to an attack by *Beauveria* fungus at the developing prepupae. Pure sand and pure peatmoss samples resulted in an elevated mortality rate due to the inability of *C. tannourinensis* larvae to form the earth cell chamber or loge as influenced directly by strictly reduced moisture levels in both cases.

Correlation analysis between different soil properties namely, pH, EC, $CaCO_3$%, P, K, Ca, Mg, Na, Fe, Zn, Mn, Cu, textural class, and organic matter content versus prepupal mortality indicated that only two properties affect prepupal survival; soil moisture and textural class (Pearson correlation = - 0.782 for moisture and 0.791 for

Table 7. Influence of different soils on the mortality and prepupae development of *Cephalcia tannourinensis* under laboratory conditions.

Soil sample*	Mean percentage*		
	Mortality	Pronymph	Eonymph
T1	27.8[a]	66.7[a]	5.6[a]
T2	27.8[a]	66.7[a]	5.6[a]
M3	100.0[c]	0.0[b]	0.0[a]
M4	38.9[a]	61.1[a]	0.0[a]
H1	83.3b[c]	16.6[b]	0.0[a]
H2	38.9[a]	61.1[a]	0.0[a]
B1	33.3[a]	55.6[a]	11.1[a]
Sand	77.8[b]	22.2[b]	0.0[a]
Peat	100.0[c]	0.0[b]	0.0[a]

T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.; *The numbers are the mean percentage of three replications. Means followed by different letters within a column are significantly different (ANOVA, P<0.05, LSD test).

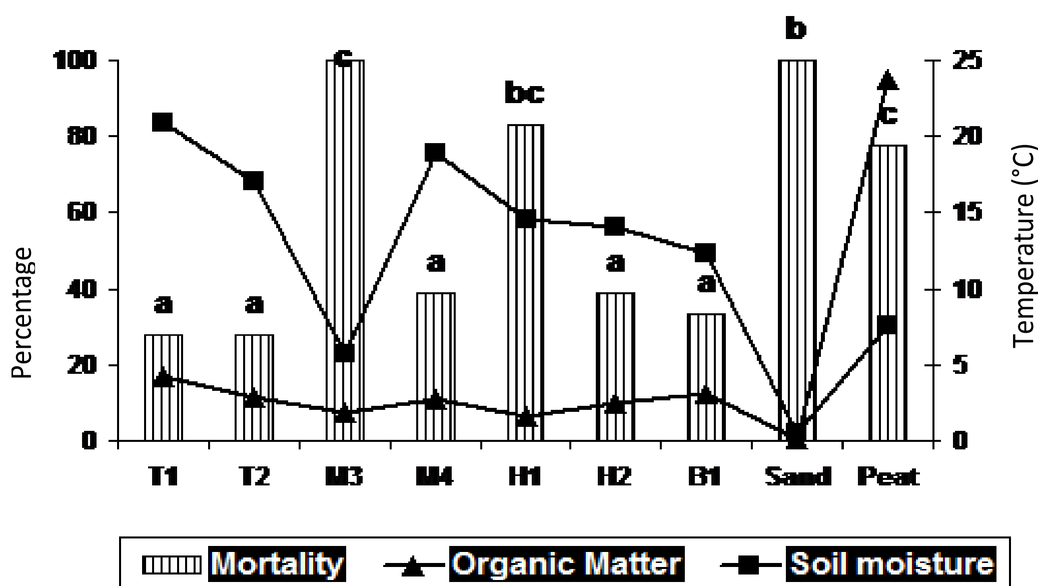

Figure 1. Influence of different soils, soil moisture content and organic matter on the mortality of *Cephalcia tannourinensis* under laboratory conditions. T = Tannourine; M = Maasser; H = Hadath EL-Jebbeh; B = Bcharry.

texture). The relationship between prepupa mortality and soil moisture is inversely related. The relationship between prepupa mortality and texture was direct and mortality increased as the soil texture changed from clayey to clay loam, peat, and sand, respectively. The highest mortality was seen in soils with low moisture content; Maasser (M3), pure sand and peat, respectively (Figure 1).

The above results demonstrated that limiting factors for the survival of *C. tannourinensis* prepupae are moisture and soil texture. Only few studies have suggested that moisture controls diapause development directly in arthropods (Tauber and Tauber, 1976). Some studies indicated that water has an essential role in post-diapause developmental phase of certain arthropods (Danks, 1987; Tauber et al., 1998). The European corn borer, *Ostrinia nubilalis,* has a two-phase diapause process, whereby changes in photoperiod terminated diapause but water was required to activate the neuro-endocrine system to allow post-diapause development

Table 8. Influence of temperature on the mortality and prepupal development of *Cephalcia tannourinensis* under laboratory conditions.

Parameter	T=20°C; n=60 mean± SE	T=10°C; n=60 mean± SE
Dead	4.0 ± 0.00[a]*	5.7 ± 0.33[a]
Pronymph	9.7 ± 1.20[a]	3.7 ± 1.20[b]
Eonymph	6.3 ± 1.20[a]	10.7 ± 0.88[a]

*Means followed by the same letter within a row are not statistically different (*t* test).

Table 9. Influence of soil moisture on the mortality and prepupal development of *Cephalcia tannourinensis* under laboratory conditions.

Parameter	M =5%; n=60 mean± SE	M =20%; n=60 mean± SE	M =40%; n=60 mean± SE
Dead	11.3 ± 1.76[c]	5.0 ± 0.57[a]	6.3 ± 0.30[ab]
Pronymph	6.3 ± 0.33a[b]	8.3 ± 0.33[c]	5.0 ± 0.00[a]
Eonymph	2.3 ± 1.45[a]	6.7 ±0.33[bc]	8.7 ± 0.33[c]

*Means followed by different letters within a row are statistically different (ANOVA, P<0.05, LSD test).

to be completed (Beck, 1967). Some studies conducted on tropical insects found that the presence or absence of moisture appears to play a major role in diapause initiation (Tauber and Tauber, 1976; Seymour, 1991).

The rate of prepupal development into eonymph was almost the same in all soils (ANOVA; F=0.792; df = 8. 26; P>0.05) whereas development into pronymph was significantly different between the different soils (ANOVA; F =9.630; df = 8. 26; P<0.0001). Pronymph was positively correlated with the moisture factor and texture (Pearson correlation = 0.814 for moisture and -0.812 for texture) under the conditions of this study.

All other factors showed no correlation with the development of pronymph. The relationship between pronymph and moisture was positive; as soil moisture increased from 5, 20 to 40%, respectively, the number of pronymph also increased. The relationship between the number of developing pronymph and texture was inversely related. The proportion of pronymph increased as the soil texture changed sequentially from sand, peat clay loam to clay.

Effect of soil temperature on the development of Prepupae of *C. tannourinensis*

Results show that the two studied temperatures that is, 10 and 20°C did not affect the mortality of *C. tannourinensis* under laboratory conditions (Table 8). Soil moisture was maintained constant at 18% throughout the experiment. A high number of pronymphs was obtained under a temperature of 20°C and was significantly different from that at 10°C. Eonymphs were not significantly different between the two temperature regimes although a higher number of eonymphs was recorded in the results at 10°C temperature. Battisti (1994a, b) demonstrated that prolonged diapause as an eonymph occurred in prepupae of *C. arvensis* just after soil penetration at temperature below 12°C. He also found that mature larvae that entered the soil at tempe-ratures above 12°C changed immediately into pronymphs and these individuals will emerge during the next spring. These findings confirm our results in that a higher number of pronymphs was obtained when mature larvae were placed in soils kept at temperature of 20°C than when placed at 10°C (Table 8).

The none significance between eonymph numbers in both temperature regimes could be attributed to an interaction between the temperature and soil moisture, since soil moisture was found to be significant with both mortality and pronymph development in the first experiment.

Effect of soil moisture on the development of Prepupae of *C. tannourinensis*

When exposed to different moisture regimes, physiological development of *C. tannourinensis* larvae showed significant differences in the number of dead, pronymps and eonymphs (Table 9). The highest number of dead larvae was recorded at 5% soil moisture level,

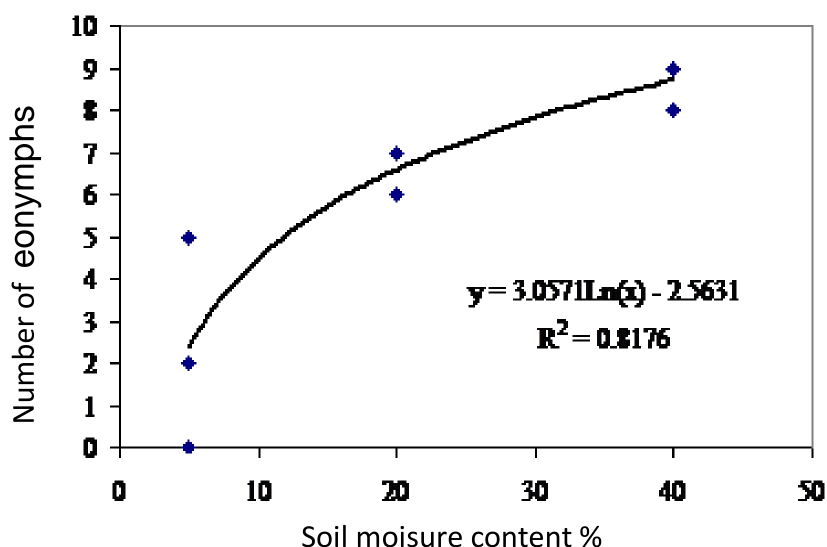

Figure 2. Logarithmic regression between the soil moisture levels and the number of larvae that developed into eonymphs at a constant temperature of 20°C.

and it was significantly higher from the 20 and 40% soil moisture regime (ANOVA, F=9.41, df=2, P<0.05). These results confirm the results of experiment 1 where a high mortality was found in the soils with low soil moisture content that is, M3, sand and peat moss.

Pronymphs were significantly higher in 20% soil moisture level as compared to the other two that is,. 5 and 40% (ANOVA, F =38, df= 2, P < 0.001). These results may be attributed to the fact that the lowest moisture level led to highest mortally rate and therefore the number of pronymphs was reduced. In the high moisture level, the number of larvae entering an annual diapause cycle was low due to the fact that a high proportion of the larvae entered a multi-annual diapause cycle reflected by the high number of eonymphs recorded (8.7 ± 0.33). The second explanation may be related to the temperature of the experimental set up which was kept constant at 20°C. In the previous experiment a higher number of pronymphs was obtained at 20°C as compared to 10°C. Therefore the interaction between temperature and soil moisture may also affect the development of prepupae. Further experiments where both the temperature and soil moisture are varying are required to demonstrate the interaction effect of these two limiting factors in the development of prepupae of *C. tannourinensis*.

ANOVA analysis of eonymphs was significantly different between the different soil moisture levels (ANOVA, F= 13.48, df=2, P<0.01). Eonymphs numbers were highest in soils with 40% moisture content followed by 20 and 5% soil moisture content, respectively.

Correlation analysis between soil moisture levels and the number of dead larvae and pronymphs respectively were not significant. However, the number of eonymphs was positively correlated with moisture levels (Figure 2). Regression analysis between soil moisture levels and the number of eonymphs was significant and is defined in the following logarithmic equation:

$$Y= -2.56 + 3.06 \ln (x)$$

Where, Y represent the number of eonymphs and x the soil moisture levels.

The equation is applicable only for soil moisture contents below 40%. The experiment showed that at 40% the earth cell chamber made by the prepupae of *C. tannourinensis* resisted well the pressure of water.

Distribution of Prepupae in the cylinders

All pronymphs and eonymphs were present in the lower 10 cm of the plastic cylinders indicating that prepupae preferred to go deep in the soil where soil moisture content would be greater than in the upper soil layers of the cylinders. Battisti (1993) found that eonymphs are usually found deeper in the soil than pronymphs which is different from the results in the plastic cylinders. One explanation could be due to the experimental setup which is not the same used by Battisti (1993) who observed the distribution of prepupae in natural conditions.

Weight analysis of pronymphs and prepupae was significantly different between the annual individuals and the multi-annual individuals (Figure 3). The results are

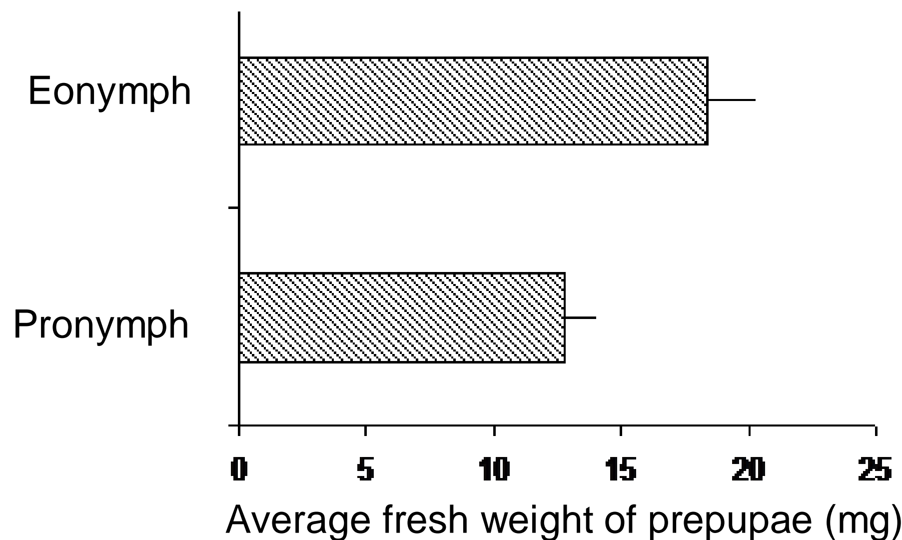

Figure 3. Average fresh weight of mature prepupae after removal from plastic cylinders in October 2007. Horizontal lines represent the standard deviations.

similar to those obtained by Battisti (1993) where biennial individuals of *C. arvensis* were heavier than annual individuals.

The difference observed in prepupal weight among annual and multiannual individuals could be related to the kind of larval feeding since, once in the soil, the prepupae construct a cell earth chamber and they do not feed. This may have a biochemical origin and can be correlated with climatic factors and larval development on the trees as suggested by Battisti and Cescatti (1994) and therefore the fate of prepupae would be determined at the feeding larval stages.

In this study, the diapausing cycles of *C. tannourinensis* were demonstrated to be related to three soil properties: soil moisture content, soil temperature and soil texture. The long life cycle and the prolonged diapause observed in *C. tannourinensis* is an example among 150 other insects belonging to different orders and show the pattern (Saulich, 2010). In many other species, however, the requirement for specific diapause-terminating conditions (Tauber and Tauber, 1976) is strict and/or fitness is considerably compromised without their intervening effect (Hodek, 1996 ; Hodek, 2002 ; Hodek, 2003). For instance, the maintenance phase, under constant laboratory conditions that were used for diapause induction and initiation, will continue until all larvae of the fly *Chymomyza costata* die, while the termination may proceed only at low temperatures (Koštál et al., 2000). In the laboratory, the maintenance and termination phases can be clearly separated in some insects by specific settings of the environmental conditions. In the field, complexity, fluctuations and linear changes of environmental conditions make the distinction more difficult.

Nevertheless, it has been shown that specific conditions/stimuli often do participate in the termination of diapause in the field, even if they are not strictly required in the laboratory. This is ecologically meaningful because the initiation of diapause may take place during very different periods of the year in different individuals of the same population. Each individual then maintains the diapause for a different time before the advent of the adversity period, which then serves as synchronizing stimulus and prevents untimely (premature) termination of diapause. Termination then takes the form of a distinct eco-physiological phase, during which diapause intensity decreases to its minimum level and subsequent resumption of direct development is enabled. Numerous examples of the effects of diapause-terminating conditions are reported in the literature. Chilling is the most common factor terminating many winter diapauses in the field (Tauber et al., 1986; Hodek, 1996; Hodek, 2002). Tanno (1970) found that freezing was a necessary factor for termination of pre-pupal diapause in the Japanese poplar sawfly *Trichiocampus populi,* both in the field and laboratory. Most of the summer diapauses and, perhaps, even some rare cases of winter diapauses, are terminated in the field by the change of photoperiodic signal (Masaki, 1980; Tauber et al., 1986, Ito, 1988). Contact with water was reported to serve as the terminating factor for summer diapauses of the larvae of the stem borer *Busseola fusca* (Okuda, 1990), and of the eggs of the chrysomelid beetle *Homichloda barkeri* (Nahrung and Merritt, 1999) (but see also the discussions in: Tauber et al., 1998; Hodek, 2003). In some crustaceans, drying of the sediment increases emergence from diapausing eggs upon r e-hydration (Arnott

and Yan, 2002).

The results obtained in these series of experiments demonstrated that the *C. tannourinensis* prepupae transformation into pronymphs and eonymphs is more related to soil moisture content and temperature. Prepupae of *C. tannourinensis* survive well in clayey, silty and silty loam soils but cannot survive or continue their development in sandy or organic soils. Soil moisture is critical for the survival of *C. tannourinenis* prepupae: soils with moisture contents lower than 10% when *C. tannourinensis* drops to the ground and enter the soil are lethal to the prepupae. Soil moisture content and soil temperature are the most limiting factors in the determination of annual diapause individuals and multi annual diapause individuals.

Tannourine Hadath El-Jebbeh and Bcharry soils constitute favorable microhabitat environment for the development of *C. tannourinensis* as compared to the Chouf cedars where the soil moisture content and organic matter are lower. The presence of an organic horizon layers enhanced the survival of *C. tannourinensis* prepupae. In these soils the water holding capacity is greater than in mineral soils and thus soil moisture remains adequate all throughout all the summer season. However, not all the diapause was explained by these three properties. Other factors related to feeding habit of the larvae and endocrinology studies are to be exploited to determine their relation to diapause. Considerable increase of *C. tannourinensis* populations has been observed at Bcharry cedar forest where a high number of pronymphs was detected indicating an increase in the temperature of soil that is favorable for their development. Suppression tactics are required if these conditions will remain prevalent.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Changes in growth and nutrient uptake in response to foliar application of sodium and calcium chloride in cowpea cultivars (*Vigna unguiculata* L. Walp)

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In this study, the effects of NaCl and $CaCl_2$ on growth and nutrients uptake of cowpea (*Vigna unguiculata* L. Walp cvs. Mouola GG; Mouola PG, Garoua GG, Garoua PG and Tsacre) were investigated. Three treatments [0 (unsprayed), 50 mM NaCl or 50 mM $CaCl_2$] were adopted, with five replications. NaCl and $CaCl_2$ were applied as foliar spray twice a week during 30 days after sowing. Application of NaCl resulted in significant decreases in plant height, dry weights of root and shoot of Garoua GG, Mouola PG and Tsacre compared to Garoua PG and Mouola GG cultivars, while those growth parameters were significantly reduced under $CaCl_2$ treatment in all cowpea cultivars except Mouola PG. K^+, Ca^{2+} and Mg^{2+} contents decreased under NaCl stress in leaves of Garoua PG and Mouola GG, but no significant changes were observed in those of Garoua GG, Mouola PG and Tsacre. The main strategy of salt tolerance in Mouola GG and Garoua PG seems to be as a result of increased osmotic adjustment through the accumulation of Na^+ in leaves while in salt sensitive Mouola PG, Garoua GG and Tsacre, the osmotic adjustment may be due to the accumulation of inorganic ions (K^+, Ca^{2+} and Mg^{2+}) in leaves, contributing to the maintenance of water uptake and cell turgor, allowing for physiological processes. In the analysis of growth parameters measured, the results suggested that Mouola GG and Garoua PG cultivars were relatively more tolerant to salinity than others, suggesting that those cultivars could be cultivated in the environment with varying salinity. $CaCl_2$ treatment significantly increased growth parameters and nutrients uptake specially Ca^{2+} in Mouola PG cultivar, suggesting that it could grow and develop on calcareous soils.

Key word: Foliar spray, growth, nutrients uptake, saline and calcareous soils, *Vigna unguiculata*.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important food legumes grown in the semi-arid tropical regions (Singh, 2003). 14.5 million hectares of land is planted to cowpea each year worldwide. Global produc-

tion of dried cowpeas in 2010 was 5.5 million metric tons (Abate et al., 2011). The top producers are the West and Central African sub regions which contribute to about 64% of the global production and an estimated production of 3 million tons of cowpea seeds produced annually (Fery, 2002). The protein content in cowpea leaves annually consumed by Africans and Asians is equivalent to 5 million tons of dry cowpea seeds, representing as much as 30% of the total food legume production in the lowland tropics (Steele et al., 1985). The economic importance of cowpea species show a number of advantages that make them particularly valuable for inclusion in many types of cropping systems (Fery, 2002). Salinity is one of the most serious abiotic stresses that lead to the deterioration of agricultural lands and reduction in crop productivity in many parts of the world especially in arid and semi-arid regions (Munns, 2002; Taffouo et al., 2010a).

The ability of vegetation to survive under higher salinity conditions is essential for the distribution of plants and agricultural grounds around the world (Yousif et al., 2010). Excessive salinity in marginal soils results from natural processes, whereas in arable land it is mostly anthropically generated, due to the progressive accumulation of the ions dissolved in irrigation water (Neumann, 1997). This later salinisation results to an alarming situation for agriculture especially in arid and semi-arid regions which about half of their surface is already affected by salt stress, to a higher or lower degree, and more than 10 million ha of agricultural land are lost every year due to salinisation (Munns and Tester, 2008). Maintaining adequate nutrient elements in the growth media under salinity is a common goal in grain legume production. Soil salinity can inhibit plant growth by a number of mechanisms such as low external water potential, toxicity of absorbed Na^+ and Cl^- ions, due to the inhibition of many enzymatic activities and different cellular processes (protein synthesis or generation of reactive oxygen species) and interference with the uptake of essential nutrients, such as K^+ and Ca^{2+} (Munns, 2002; Grigore et al., 2011). The severity of each of these factors to plant growth depends on the plant genotype and environmental conditions (Zadeh and Naeini, 2007). The nutritional imbalance in plants caused by salt stress may result from the effect of salinity on nutrient availability, competitive uptake and transport or partitioning within the plant (Munns and Tester, 2008).

NaCl toxicity which is the major form of salt in most saline soils enhances the Na^+ and Cl^- contents and consequently affects the absorption of other mineral elements (Greenway and Munns, 1980). It is stated that high levels of Na^+ inhibits Ca^{2+} and K^+ absorption, which results in a Na^+/K^+ antagonism (Turan et al., 2007). The fundamental mechanisms of salt tolerance in salt tolerant plants seem to be mostly dependent on their capacities to sequestrate toxic ions (Na^+, Cl^-) in the vacuoles and to accumulate compatible osmotica in the cytoplasm (Munns, 2002). Ca^{2+} plays a vital role in many physiological processes such as membrane structure and stomatal functioning cell division, cell wall synthesis and osmoregulation, which influence growth and responses towards environmental stresses (Kusvuran, 2012). The maintenance of an adequate supply of Ca^{2+} in saline soil solutions is an important factor in controlling the severity of specific ion toxicities, particularly in crops which are susceptible to Na^+ and Cl^- injuries (Grattan and Grieve, 1999). Under this condition, the ameliorative action of supplemental Ca^{2+} is crucial to alleviate high salinity stress. Ca^{2+} has a role in building salt tolerance in plants (Amuthavalli et al., 2012). Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating a high K^+/Na^+ selectivity (Liu and Zhu, 1998). Other beneficial effects that $CaCl_2$ has on plant physiology include membrane permeability and reduction in Na^+ concentration (Amuthavalli et al., 2012). Studies about the use of foliar organic or inorganic substances application on vegetable species are scarcely reported in the literature, however, some investigations have been conducted in groundnut (Lee, 1990), cucumber (Ozdamar Unlu et al., 2011), cowpea (Khalil and Mandurah, 1989), watermelon (Silva-Matos et al., 2012), pepper (Karakurt et al., 2009), rice (Kaur and Singh, 1987), white gourd (Ali et al., 2010) and common bean (Rahman et al., 2014), with promising results. However, the effect of foliar spray of NaCl and $CaCl_2$ on the growth and nutrient uptake of cowpea seedlings has been poorly quantified.

The objective of this study, therefore, was to investigate the effect of foliar application of NaCl and $CaCl_2$ on growth and nutrients accumulation and determine the main strategy of cowpea salt tolerance and identification of salt tolerant cultivars which could be grown in saline or calcareous soils.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of cowpea (*V. unguiculata* L. Walp) cvs. Mouola GG; Mouola PG, Garoua GG, Garoua PG and Tsacre provided by the breeding program of the Agronomic Institute for Research and Development of Cameroon were used in this study. The experiment was conducted from July 2011 to December 2011 at the Faculty of Science, University of Douala, Cameroon. Seeds with similar sizes and weights were washed with distilled water. The seeds were then sterilized for 20 min using sodium hypochlorite 3% (w/v) and rinsed 5 times using distilled water. Three days after germination, the primordial leaves were established. The seedlings were transferred

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to pots with 750 cm^3 capacity filled with 0.7 kg of sterilized quartz sand as substrate. The pots were kept in the laboratory (temperature: 26°C/20°C, light: 5000 lux for 12 h alternating periods and relative humidity of 61%) and supplied every three days with modified Wacquant (1974) nutrient solution containing 0.4 mM KNO_3, 0.2 mM KH_2PO_4 and 0.4 mM $MgSO_4$.

Treatments

A completely randomized block design with three treatments [0 (unsprayed), 50 mM NaCl or 50 mM $CaCl_2$] was adopted, with five replications of five seedlings each, with a total of 75 seedlings of all cultivars. The exogenous substances (NaCl and $CaCl_2$) were diluted using distilled water to get two different treatment solutions. Cowpea seedlings were sprayed with 30 ml NaCl or $CaCl_2$ twice a week until the end of the experiment (30 days) with a hand sprayer in the evening late hours to avoid dehydration at midday. Five randomly chosen plants from each cultivar and treatment were harvested after 30 days and used for physiological analysis.

Plant height and dry weights

The plant heights were measured every two days in each group of seedlings. Plant samples were harvested after 30 days and the harvested leaves, stems and roots were weighed to determine their fresh weights (FW). Dry weights (DW) were determined after the leaves, stems and roots were dried in an oven at 70°C for 72 h.

Nutrient analysis

For chemical analysis, the plant samples were dried in an oven for 72 h at 70°C. The Na^+ and K^+ contents in plants were determined according to Savouré (1980) methodology, while Mg^{2+}, Ca^{2+}, P and N contents were determined using the method described by Taffouo (1994).

Statistical analysis

All analyses were carried out in accordance with completely randomized design. Data were statistically analysed by analysis of variance using the SPSS software package (SPSS 10.0 for Windows 2001). The statistical differences between the experimental and control groups were established by the ANOVA. Each data point was the mean of five replicates (n = 5) and comparison between means was done using the Duncan's multiple range tests at 5% probability level.

RESULTS

Effect of foliar application of NaCl and $CaCl_2$ on dry weights of cowpea organs

The growth parameters such as shoot and root dry weights were measured after spraying exogenous substances (NaCl or $CaCl_2$) on leaves of cowpea cultivars (Figure 1A and B). The present study shows that NaCl treatment significantly inhibited the shoot dry weights of Garoua GG, Mouola PG and Tsacre cultivars while there were no significant differences in those of Garoua PG and Mouola GG, as compared with the control groups (Figure 1A). The application of NaCl did not cause significant changes in root dry weights of all cowpea cultivars, except in Tsacre (Figure 1B). The application of $CaCl_2$ significantly reduced the shoot dry weights of Garoua GG, Garoua PG, Mouola GG and Tsacre but no significant changes were observed in Mouola PG cultivars (Figure 1A), as compared with the control groups, while the root dry weights were not significantly reduced for these cultivars except in Tsacre (Figure 1B).

Effect of foliar application of NaCl and $CaCl_2$ on plant height

The present study shows that the foliar spray of NaCl and $CaCl_2$ caused a significant reduction in the plant height of the cowpea cultivars as compared to the control, except for Mouola GG (Figure 2). The magnitude of plant height reduction was highly dependent upon cowpea cultivars. The plant heights of Tsacre and Garoua GG cultivars showed higher decreases (44.87 and 39.28% respectively, compared to the control) than those of Mouola PG, Garoua PG and Mouola GG (21.94, 19.83 and 0.06%, respectively) under NaCl application (Figure 2).

The plant heights of all the cowpea cultivars decreased under $CaCl_2$ application. Cultivars Mouola PG, Garoua GG and Mouola GG were the least affected, with decreases of 17.98, 19.42 and 24.47%, respectively, compared to the control. Tsacre and Garoua PG cultivars showed the highest reductions in plant heights, with decreases of 44.00 and 33.45%, respectively (Figure 2).

Effect of foliar application of NaCl on nutrient uptake

The effects of foliar spray of NaCl on nutrient uptake were examined in the leaves of cowpea cultivars (Table 1). All cowpea cultivars accumulated Na^+ ($P<0.05$) in leaves compared to the control. The concentrations of Na^+ in leaves of Mouola GG and Garoua PG were higher (17.8 and 12.4 mg g^{-1} DW) than those of Mouola PG, Garoua GG and Tsacre (11.5, 10.5 and 10.1 mg g^{-1} DW), respectively. The Na^+/K^+ ratio in the leaves of Mouola GG and Garoua PG were slightly higher (0.70 and 0.59, respectively, compared to the control) than those of Mouola PG, Garoua GG and Tsacre (0.40, 0.30 and 0.37 respectively). The K^+, Ca^{2+} and Mg^{2+} concentrations in leaves of Mouola GG and Garoua PG cultivars were markedly decreased on salt treatment; however, those elements in leaves of Garoua GG, Mouola PG and Tsacre were not changed by NaCl application, as compared to the control (Table 1). On the other hand, N and P uptake of Garoua GG, Mouola PG and Tsacre cultivars were significantly decreased on salt treatment; however, those elements in leaves of Mouola GG and Garoua PG cultivars were not affected by NaCl application as compared to the control (Table 1).

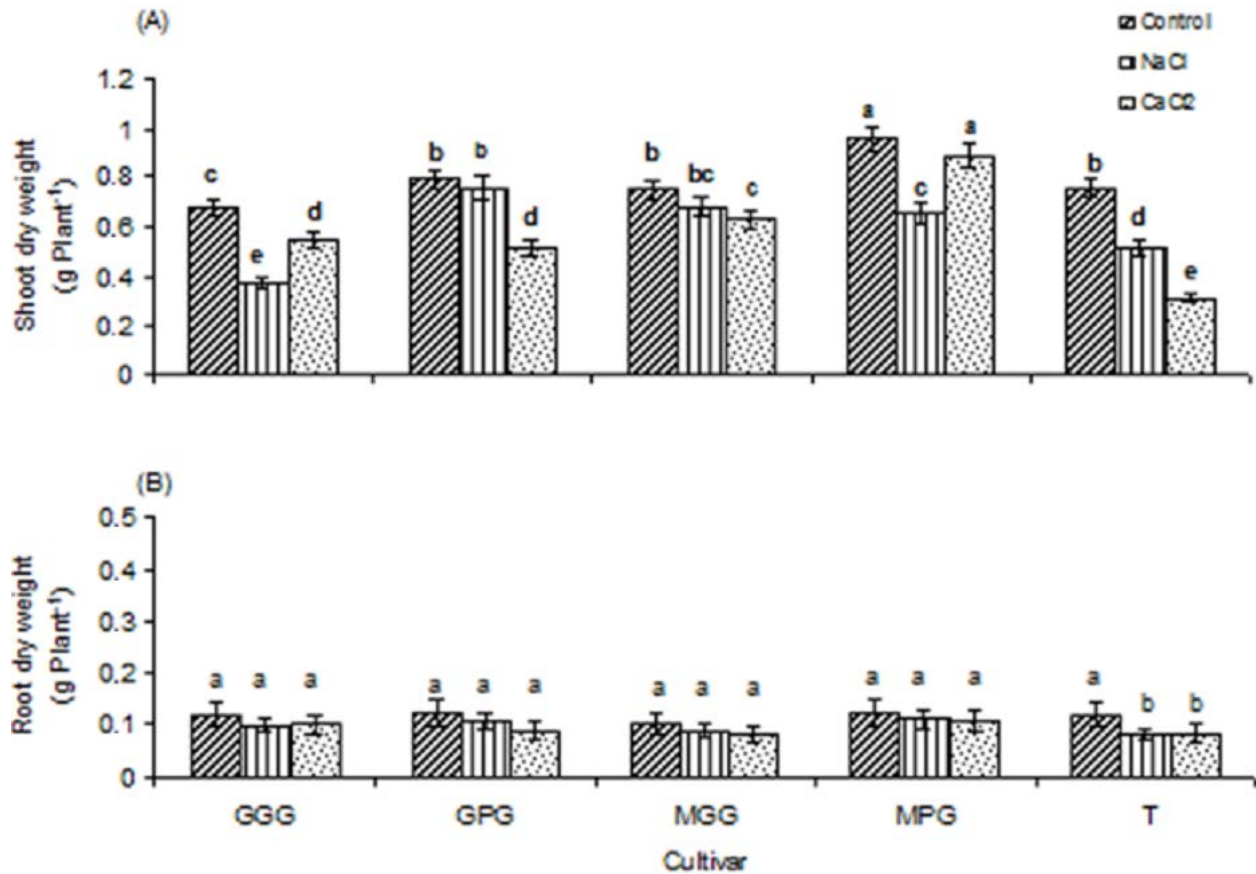

Figure 1. Shoot dry weight (A), root dry weight (B) of cowpea cultivars as affected by foliar application of NaCl and $CaCl_2$ during vegetative stage. Values are given as mean±SD, n = 5 for each cultivar; Garoua GG (GGG), Garoua PG (GPG), Mouola GG (MGG), Mouola PG (MPG) and Tsacre (T). Values of each bar followed by the same letter indicate no significant difference (P < 0.05) according to Duncan's test.

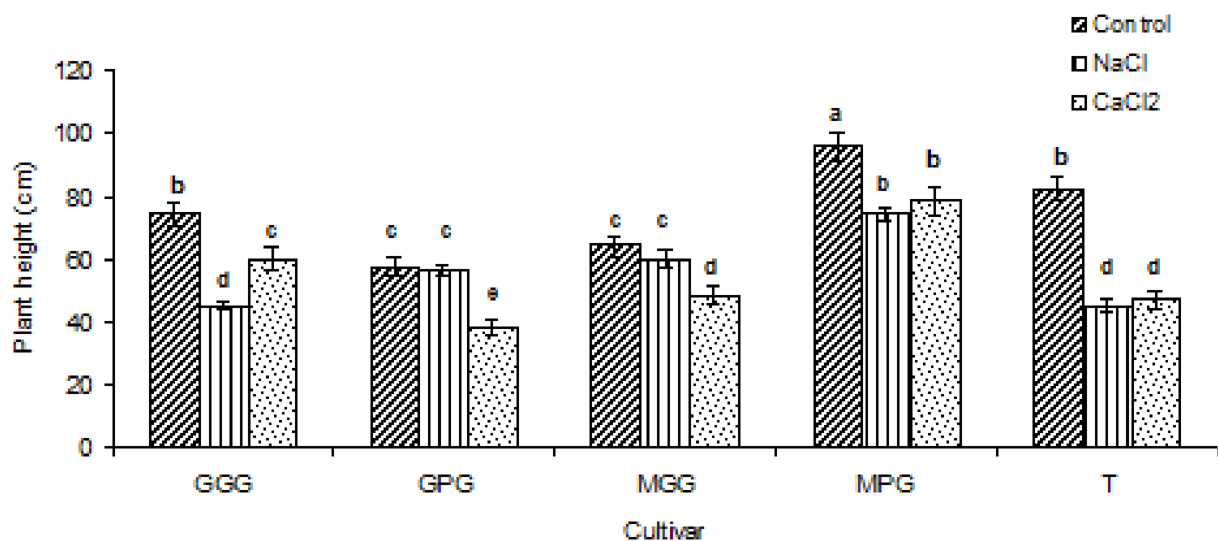

Figure 2. Plant height of cowpea cultivars as affected by foliar application of NaCl and $CaCl_2$ during vegetative stage. Values are given as mean±SD, n = 5 for each cultivar; Garoua GG (GGG), Garoua PG (GPG), Mouola GG (MGG), Mouola PG (MPG) and Tsacre (T). Values of each bar followed by the same letter indicate no significant difference (P < 0.05) according to Duncan's test.

Table 1. Effect of foliar application of NaCl on nutrient contents in leaves of cowpea cultivars during vegetative stage.

Cultivar	NaCl concentration (mM)	Nutrient concentrations (mg g^{-1} DW)						Na^+/K^+ ratio
		Na^+	K^+	Ca^{2+}	Mg^{2+}	N	P	
Garoua GG	0	4.1±0.2^d	38.7±1.3^a	16.7±1.7^{ab}	6.5±0.3^a	40.6±1.1^a	6.1±0.4^a	0.11
	50	10.9±0.4^c	36.5±1.4^a	15.3±0.6^b	5.1±0.1^a	26.4±0.4^c	4.2±0.3^b	0.30
Garoua PG	0	3.2±0.1^d	30.3±0.8^{bc}	19.1±1.1^a	7.4±0.5^a	42.2±2.3^a	6.3±0.4^a	0.11
	50	12.4±0.2^b	20.9±0.2^d	11.2±0.6^c	2.2±0.2^b	41.5±1.3^a	5.8±0.6^a	0.59
Mouola GG	0	2.9±0.1^d	33.8±1.6^b	22.8±1.3^a	6.4±0.7^a	40.7±0.7^a	6.1±0.7^a	0.09
	50	17.8±0.5^a	25.5±1.9^c	13.5±1.2^{bc}	2.1±0.3^b	38.6±1.3^a	5.3±0.4^a	0.70
Mouola PG	0	3.5±0.3^d	32.4±1.2^b	18.5±1.8^a	5.8±0.2^a	41.2±3.0^a	6.6±0.2^a	0.11
	50	11.5±0.4^{bc}	29.0±0.5^b	16.9±0.2^{ab}	4.2±0.7^a	33.6±2.1^b	4.6±0.1^b	0.40
Tsacre	0	2.9±0.7^d	29.5±1.9^b	21.7±2.1^a	7.5±0.8^a	43.9±1.1^a	6.7±0.6^a	0.10
	50	10.1±0.2^c	27.2±1.4^{bc}	19.6±1.6^a	6.3±0.4^a	32.8±2.0^b	4.1±0.2^b	0.37

Data represent mean ± SE (n = 5); within columns, means followed by the same letter are not significantly different (*p*<0.05) by Duncan test.

Table 2. Effect of foliar application of $CaCl_2$ on nutrient contents in leaves of cowpea cultivars during vegetative stage.

Cultivar	$CaCl_2$ concentration (mM)	Nutrient concentrations (mg g^{-1} DW)						Na^+/Ca^{2+} ratio
		Na^+	K^+	Ca^{2+}	Mg^{2+}	N	P	
Garoua GG	0	8.7±0.2^b	28.7±1.3^b	20.9±1.7^b	6.5±0.3^a	40.6±1.1^b	6.1±0.4^a	0.41
	50	4.1±0.7^c	26.3±2.9^c	19.1±0.2^b	4.6±0.2^b	39.1±0.4^b	5.6±0.3^a	0.21
Garoua PG	0	7.1±0.1^b	30.3±0.8^b	19.1±1.1^b	7.4±0.5^a	41.2±2.3^b	6.3±0.4^a	0.37
	50	3.2±0.4^c	33.0±1.9^a	17.8±1.5^{bc}	6.6±0.9^a	40.8±3.1^b	5.3±0.3^a	0.18
Mouola GG	0	13.1±0.1^a	25.8±1.6^c	22.8±1.3^b	6.4±0.7^a	40.7±0.7^b	6.1±0.7^a	0.57
	50	2.9±0.5^c	24.9±0.3^c	24.1±0.7^b	7.1±0.8^a	41.9±1.6^b	5.2±0.7^a	0.12
Mouola PG	0	13.5±0.3^{ab}	25.4±1.2^c	18.5±1.8^c	5.8±0.2^a	41.2±3.0^b	6.6±0.2^a	0.73
	50	8.5±0.3^c	26.9±2.2^c	26.2±1.6^a	6.0±1.4^a	40.4±2.1^b	5.1±0.4^a	0.32
Tsacre	0	6.2±0.7^b	26.5±1.9^c	21.7±2.1^b	7.5±0.8^a	43.9±1.1^a	6.7±0.6^a	0.29
	50	2.9±0.3^c	24.1±1.6^d	23.2±0.2^b	5.2±1.1^b	41.3±1.1^b	4.4±0.5^b	0.13

Data represent mean ± SE (n = 5); within columns, means followed by the same letter are not significantly different (*p*<0.05) by Duncan test.

Effect of foliar application of $CaCl_2$ on nutrient uptake

Changes in nutrient contents in the leaves of cowpea cultivars subjected to foliar application of $CaCl_2$ during seedling stages are presented in Table 2. As can be shown by statistical analyses (P<0.05), Na^+ content was significantly decreased by $CaCl_2$ application in the leaves of all cowpea cultivars. In this study, $CaCl_2$ treatment decreased significantly K^+ and Mg^{2+} contents in the leaves of Garoua GG and Tsacre cultivars while those of Mouola GG and Mouola PG did not significantly affected. On the other hand, Ca^{2+} content was significantly increased in the leaves of Mouola PG by the application of Ca^{2+}, as compared to the control and the other cultivars studied (Table 2). The Na^+/Ca^{2+} ratio in the leaves of Mouola PG was slightly higher (0.32) than those of Garoua GG, Garoua PG, Mouola GG and Tsacre cultivars (0.21, 0.18, 0.12 and 0.13), respectively as compared to the control. $CaCl_2$ application decreased significantly N and P contents in the leaves of Tsacre cultivar (Table 2).

DISCUSSION

In the present study, NaCl treatment inhibited significantly the shoot dry weights of Garoua GG, Mouola PG and Tsacre cultivars while there were no significant differences in those of Garoua PG and Mouola GG as compared to the control (Figure 1A). Crop responses to foliar application have been mixed either positive or negative responses depending on crop species and nutrients applied. Numerous studies conducted on foliar fertilization with N, P, K and S during early vegetative growth stages or late reproductive growth stages showed inconsistent growth and grain yields increases (Parker and Boswel, 1980; Haq and Mallarino, 2000; Nelson et al., 2005). These authors reported that leaf damage due to foliar fertilization sometimes were severe enough to cause growth and yield reductions. Reduced seedling growth under saline conditions has also been reported by Huang and Redmann (1995) on barley, Taffouo et al. (2010a) on bambara groundnut and Erum Mukhtar et al. (2013) on canola, respectively. The effect of salinity

application on seedling growth found a reduction in shoot dry weight that may be due to toxic effects of NaCl used as well as unbalanced nutrient uptake by the seedlings (Grigore et al., 2011). According to Jaleel et al. (2009), the reduction of plant growth under salinity is the result of the alteration of many physiological activities in the plant, such as photosynthetic activity, mineral uptake and antioxidant activity. Rahman et al. (2008) reported that salinity depressed shoot than root dry weights and increased root/shoot ratio. Shoot dry weight of all cowpea cultivars (Figure 1A) was significantly reduced with $CaCl_2$ treatment except in Mouola PG compared to control, while the root dry weight was not significantly reduced for these cultivars except in Tsacre (Figure 2A). According to Levitt (1980), Mouola PG cultivar can be considered as calciophile plants that are able to grow and develop on calcareous soils at high Ca^{2+} levels. In those obligate calciophile plants, the Ca^{2+} plays an essential role in processes that preserve the structural and functional integrity of plant cell membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion-exchange behaviour as well as cell wall enzyme activities (Marschner, 1995). In this study, the shoot dry weights of Garoua GG, Garoua PG, Mouola GG and Tsacre cultivars were significantly ($P<0.05$) reduced with $CaCl_2$ application; those cultivars appear to be typical calciophobe plants. Similar reductions in the dry weights were observed in *Gossypium hirsutum* plants growing under $CaCl_2$ conditions (Amuthavalli et al., 2012). In the presence of excess Ca^{2+}, the calciophobe plants would absorb considerable Ca^{2+} at the expensive of other ions, and would therefore suffer from a deficiency. This implies that the Ca^{2+} resistance of the calciophile plants is due to avoidance of a secondary deficiency stress by Ca^{2+} exclusion (Levitt, 1980).

The present study shows that the foliar application of $NaCl_2$ caused a significant reduction in the plant heights of the cowpea cultivars as compared to the control, except for Mouola GG (Figure 2). Numerous studies have reported the reduction of plant heights stimulated by NaCl salinity (Khan et al., 2000; Zadeh and Naeini, 2007). In this work, $CaCl_2$ application caused a significant reduction in the plant heights of all the cowpea cultivars as compared to the control (Figure 2). Similar observa-tions for plant height reductions by $CaCl_2$ application were reported in *G. hirsutum* (Amuthavalli et al., 2012). For these cultivars, the $CaCl_2$ may reduce the growth by upsetting water and nutritional balance of the plant (Al-Khateeb, 2006).

In this research, the plant height, the dry weight of roots and shoot of the Garoua GG, Mouola PG and Tsacre showed higher decreases compared to those of Mouola GG and Garoua PG cultivars after 4 weeks of NaCl application (Figures 1 and 2). These results demonstrate that Garoua GG, Mouola PG and Tsacre cultivars, in common with certain other leguminous plant (e.g. beans), are highly sensitive to salt with severe effects even at 50 mM NaCl (Levitt, 1980). As stated by Munns (2002), the reduction of plant growth under saline conditions may either be due to decreased availability of water or to the toxicity of $NaCl_2$. It can inhibit plant growth by a range of mechanisms, including low external water potential, ion toxicity and interference with the uptake of nutrients (Salam et al., 2011). According to Yousif et al. (2010) the growth of glycophytes decreases with salinity, while that of halophytes improves. In this work, the growth of Garoua PG and Mouola GG cultivars were less affected under NaCl application, agreeing with previous data reported in *Ceriops roxburghiana*, *Phaseolus adenanthus*, *Lagenaria siceraria, Vigna subterranea*, *Tetragonia tetragonioides* and *Oryza sativa* genotypes described as salt-tolerant (Rajest et al., 1998; Taffouo et al., 2008; 2010b; Yousif et al., 2010; Mehede et al., 2014). There are a great number of plant species which are regarded as salt tolerant, the most competitive being those that are able to become established, grow to maturity and survive until they are able to reproduce (Turan et al., 2007).

In this study, NaCl application increased significantly Na^+ concentration and reduced K^+, Ca^{2+} and Mg^{2+} contents in the leaves of Mouola GG and Garoua PG cultivars, however, those elements in the leaves of Garoua GG, Mouola PG and Tsacre were not changed by NaCl Treatment (Table 1). The increase of Na^+ accumulation in Mouola GG and Garoua PG cultivars was associated with reduced K^+, Mg^{2+} and Ca^{2+}, indicating a restriction in the uptake of these nutrients, as noted in other salt tolerant plants (Sagir et al., 2002; Yousif et al., 2010). Under saline conditions, tolerant plants tend to take up and accumulate Na^+ in their vacuoles and use it as an osmoticum (Glenn and Brown, 1999). Similar outcome were obtained earlier by Al-Khateeb (2006) and Turan et al. (2007). Salinity is known to significantly reduce K^+ uptake related with reduce intracellular K^+ concentration especially in the vacuolar pool (Cuin et al., 2003). The decrease in K^+ concentration under NaCl application may be due to the competition of Na^+ with uptake of K^+, resulting in Na^+/K^+ antagonism (Carvajal et al., 2000; Turan et al., 2007). High Na^+ levels in the external medium greatly reduce the physiochemical activity of the dissolved Ca^{2+} and may thus displace Ca^{2+} from the plasma membrane of the root cells (Cramer et al., 1985). In this study, the uptake of N by Garoua GG, Mouola PG and Tsacre cultivars was decreased and that of Garoua PG and Mouola GG was not affected by NaCl application (Table 1). According to Parida and Das (2004), salinity reduces N uptake in many plants and this is attributed to antagonism between NO_3^- and Cl^-. In this work, the N uptake of Garoua PG and Mouola GG cultivars was not affected by NaCl application. According to Hu and Schmidhalter (2005), N is an essential nutrient element that plants require in the largest amounts for the biosynthesis of nitrogenous organic solutes in plants; therefore N deficiency inhibits plant growth. Adequate

supply of N is beneficial for carbohydrate and protein metabolism that promotes cell division and enlargement resulting in higher yield (Shehu et al., 2010). The results showed that NaCl application decreased P concentration in leaves of all cowpea cultivar (Table 1). Grattan and Grieve (1999) reported that P concentration in plants depended on the species, growth conditions and cultivar.

Changes in nutrient contents in leaves of cowpea cultivars subjected to foliar applied $CaCl_2$ during seedling stage are presented in Table 2. As can be shown by statistical analyses ($P<0.05$), Na^+ content was significantly decreased by $CaCl_2$ application in leaves of all cowpea cultivars. Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating a high K^+/Na^+ selectivity (Liu and Zhu, 1998). Other beneficial effects that $CaCl_2$ has on plant physiology include membrane permeability and reduction in Na^+ concentration (Amuthavalli et al., 2012). According to Arshi et al. (2010) the Ca^{2+} ameliorated the deleterious effects of NaCl stress and stimulated the plant metabolism and growth when application of NaCl and $CaCl_2$ was combined than with the NaCl treatment alone. In this study, $CaCl_2$ treatment showed significant increase of Ca^{2+} in the leaves of Mouola PG (Table 2). Arshi et al. (2010) found significant increase of Ca^{2+} concentration in plant parts of *Cichorium intybus* under $CaCl_2$ application. $CaCl_{2,}$ as compared to $NaCl_2$ treatment, which may be necessary for the osmotic adjustment by accumulation of inorganic ions (K^+, Ca^{2+} and Mg^{2+}) in cowpea leaves, contributing to the maintenance of water uptake and cell turgor, allowing for physiological processes like stomatal conductance, photosynthesis and cell expansion (Munns, 2002).

In conclusion, the growth of Mouola GG and Garoua PG cultivars were less affected under NaCl application but those of Mouola PG, Garoua GG and Tsacre were markedly decreased, indicating that Mouola GG and Garoua PG cultivars are salt tolerant. The main strategy of salt tolerance in Mouola GG and Garoua PG seems to be as a result of increased osmotic adjustment through the accumulation of Na^+ in leaves while in salt sensitive Mouola PG, Garoua GG and Tsacre, the osmotic adjustment may be due to the accumulation of inorganic ions (K^+, Ca^{2+} and Mg^{2+}) in leaves, contributing to the maintenance of water uptake and cell turgor, allowing for physiological processes. The application of $CaCl_2$ ameliorated the deleterious effects of NaCl stress and stimulated the plant growth by reducing significantly Na^+ content in the leaves of all cowpea cultivars. $CaCl_2$ treatment significantly increased growth parameters and nutrients uptake especially Ca^{2+} in Mouola PG. This cultivar may be a calciophile plant that is able to grow and develop on calcareous soils. In the analysis of growth parameters measured, the results suggested that Mouola GG and Garoua PG cultivars were relatively more tolerant to salinity than others, suggesting that those cultivars could be cultivated in the environment with varying salinity.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Recycling of vinasse in ethanol fermentation and application in Egyptian distillery factories

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Vinasse production is one of the most significant and challenging issues in the industrial production of ethanol due to pollution problems. In this study, vinasse obtained from ethanol by fermentation of sugarcane molasses was used instead of water to prepare the fermentation medium. *Saccharomyces cerevisiae* F-727 was selected from 10 tested yeast strains. Nutritional requirements of nitrogen, phosphorous and magnesium, as well as the effect of inoculum size were studied. There was a decrease in required nutrients when vinasse was used instead of fresh water; and 50% yield was achieved in fresh water together with an improved efficiency of the fermentation.

Key words: Vinasse, ethanol fermentation, *Saccharomyces cerevisiae.*

INTRODUCTION

The disposal of vinasse, the major effluent from the ethanol industry, represents a major environmental problem for the ethanol industry. This black liquid produced 10 to 15 times greater than the ethanol itself is a mixture of water and organic and inorganic compounds. These compounds remain after different steps involving sugar cane production and processing. These hazardous substances cause the vinasse to have a very high biological oxygen demand (BOD), ranging from 30-40,000 (Voegele, 2009). The inadequate and indiscriminate disposal of sugarcane vinasse in soils and water bodies has received much attention since decades ago, due to environmental problems associated to this practice (Aparecida et al., 2013).

Research has demonstrated that vinasse disposal in river basins alone is not a convenient disposal solution. Due to its high B.O.D., this material can cause damage to aquatic life, especially when dumped in large volumes. Some of the existing methods for the disposal of vinasse are direct land application and methane production (Baez-Smith, 2006). If vinasse is discharged on land, the alkalinity of the soil is reduced, leading to destruction of crops, deficiency of manganese in the soil and inhibition of seed germination. Also, the concentration-incineration of vinasse is the only system that can provide a satisfactory solution to the pollution problem; its only draw-back is high cost (Aparecida et al., 2013). Due to the large quantities of vinasse produced, alternative treatments and uses have been developed, such as recycling of vinasse in fermentation, fertirrigation,

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Table 1. *Screening S. cerevisiae* strains cultivated on sugar cane molasses medium diluted with 50% vinasse instead of water.

Yeast strain	EtOH (%)	RS (%)	FE (%)
F-707	9.2	3.8	75.4
FA-91	9.7	3.3	79.5
FF-725	9.9	2.9	81.2
F-235	9.5	3.4	77.9
F-25	9.7	3.2	79.5
FK-727	10.5	2.3	86.1
FC-620	9.9	2.8	81.2
FH-620	9.7	3.3	79.5
FAT-12	9.3	3.6	76.2
F-514	10.4	2.3	85.3

EtOH, Ethanol yield; RS, residual sugars; FE, fermentation efficiency.

concentration by evaporation,and yeast and energy (Ryznar-Luty et al., 2008; Karlsson et al., 2013). No one has found convenient and economical disposal solution for vinasse. In this paper, we worked on the alcoholic fermentation of sugar cane molasses and studied the possibility of applying vinasse, instead of water in the preparation of the fermentation medium. We applied different vinasse percentages in the medium.

MATERIALS AND METHODS

Sugarcane molasses

Sugarcane molasses produced by Egyptian Sugar and Integrated Industries Company is used as carbon source for ethanol production in the distillation factories in El-Hawamdia-Giza- Egypt.

Vinasse

Vinasse containing 8.6% total solids and 1.34% fermentable sugars was taken from the output of distillery factories, El-Hawamdia-Egyptian Sugar and Integrated Industries Company, Giza- Egypt.

Yeast strains

S. cerevisiae strains were obtained from Microbial Chemistry Department National Research Centre, Dokki, Cairo Egypt. The strains were subcultured on yeast extract, malt extract, peptone agar (YMPA) medium and preserved in refrigerator at 4°C

Inoculums preparation

100 ml of medium (g /L) consisting of malt extract (3), yeast extract (3), peptone (5) and sucrose (30) was poured into 250 ml sterile conical flasks and steam sterilized at 121°C for 15 min; it was cooled to room temperature, then inoculated with a loop of the selected yeast strains and incubated statistically at 34°C for 24 h. The growth yeast containing 4 x10^6 CFU/ml was used to inoculate the experimental flasks at 10% (v/v).

Experimental

All laboratory studies were carried in 500 ml capacity conical flasks that contained 200 ml molasses fermentation medium with 20% fermentable sugars, non- adjustable pH at 36°C.

Screening of yeast strains

Ten (10) yeast strains of *S. cervisiae* (Table 1) were cultured in diluted molasses medium (20% w/v fermentable sugars); no nutrients were added, to select the proper strains.

Effect of replacing water with varying levels of vinasse

The selected yeast strain was cultured in molasses medium diluted with vinasse instead of water at different levels (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) to study the effect of vinasse level on ethanol yield and fermentation efficiency.

Effect of nitrogen source and level

Three nitrogen sources (urea, ammonium sulfate and diammonium phosphate) were introduced individually in molasses medium diluted with different levels of vinasse to the equivalent of nitrogen at three concentrations each (100, 150 and 200 ppm) to study their effect on ethanol yield and fermentation efficiency in sugar cane molasses medium.

Effect of orthophosphoric acid supplementation

Varying concentrations of orthophosphoric acid (25-75 ppm) were added to the fermentation.

Effect of magnesium sulfate supplementation

Varying concentrations of magnesium sulfate (15-45 ppm) were added to the fermentation media under the applied concentrations of urea and orthophosphoric acid.

Effect of inoculums size

Inoculums sizes ranging from 10-25% v/v were applied to study their effect on the final ethanol yield and FE in molasses medium diluted with different vinasse levels instead of water.

Effect of fermentation time

Varying percents of vinasse under optimized parameters of urea, orthophosphoric acid and magnesium sulfate and inoculum size were applied to investigate their effect on fermentation period for maximum ethanol production.

Fermentation process

Batch culture system was employed for the fermentation of diluted molasses with varying levels of vinasse instead of water in fermenters having working volume of 65 m^3. They were supplemented with the parameters to be optimized, but afterwards a diluted molasses (20% fermentable sugars) was fed to the fermenters to enable yeast cells to utilize sugars in the molasses for conversion into ethanol. Batch molasses was adjusted so that fermenter vessels were filled to working capacity (65 m^3), and then allowed to achieve complete fermentation.

Table 2. Effect of recycling varying vinasse amounts in sugar cane molasses medium on the alcoholic fermentation yield and efficiency.

Vinasse (%, v/v)	EtOH (%)	RS (%)	FE (%)
0	11	1.8	100
10	11	1.9	100
20	11.1	1.9	100.9
30	11	2.1	100
40	10.7	2.2	97.3
50	10.5	2.3	95.5
60	10.2	2.5	92.7
70	9.6	3	87.3
80	9.3	3.8	84.6
90	8.9	4.4	80.9
100	8..5	4.9	77.3

EtOH, Ethanol yield; RS, residual sugars; FE, fermentation efficiency.

Analytical procedures

The sugar concentration was determined by rapid method. 5 ml of fermented sample was taken and dissolved in 100 ml of distilled water and mixed with 5 ml of conc. HCL acid; it was heated at 70°C for a period of 10 min. The obtained sample was neutralized by adding NaOH, prepared up to 1000 ml and transferred into burette solution. 5 ml of Fehling A and 5 ml of Fehling B were taken and mixed with 10 to 15 ml of distilled water in a conical flask and methylene blue indicator was added. The conical flask solution was titrated with burette solution in boiling conditions until disappearance of blue color. The sugar concentration was calculated by using the formula: (sugar concentration (gm/l) = [(dilution factor x Fehling factor) / titrate value] x 100). Cells count (CFU) were determined using microscope with the help of haemocytometer. Cell viability was checked by using methylene blue indicator. The dead cells were stained with blue indicator while viable cells remained uncolored. Ethanol yield (EtOH %v/v) of the fermented samples was measured by ebulliometer approved in distillation factories (Fadel et al., 2013): theoretical yield (total reducing sugars g/l x 0.51 x 0.79) multiplied by 100. The relative fermentation efficiency (RF) of fermentation process determined the percent of ethanol yield when vinasse was used in relation to the percent of ethanol yield in the molasses medium without diluted vinasse (100%).

Reproducibility of the results

All experiments were run at least three times and all reported data are given in mean values.

RESULTS AND DISCUSSION

Selection of yeast strain

Yeast strains are limiting factor in fermentation process. As a result, high osmo-tolerant *S. cerevisiae* strains are needed to ferment high concentrate molasses. This would save water for molasses dilution and reduce vinasse produced (Ingledew and Bellissimi, 2012). Ten (10) yeast strains of *S. cerevisiae* were screened in molasses medium diluted with 50% vinasse instead of water to select the most tolerant (Table 1). Data show that S*. cerevisiae* F-727 and F-514 are more efficient than the other tested strains for producing ethanol in molasses medium diluted with vinasse; they produced 10.8% v/v and 10.4 v/v, respectively. Fadel (2013) showed that the selection of yeast strain is one of the solutions to the problem of vinasse. This is because the selection of higher ethanol yielding yeast strain and optimization of the fermentation parameters improved both yield and economics of the fermentation process (Fadel et al., 2013). Research on ethanol fermentation, including the search for efficient *S. cerevisiae* strains has been on for years in order to lower down the cost of production. Desired yeast strains have the special property of possessing particularly efficient aerobic and anaerobic metabolic capabilities, making them high ethanol producers. They could also possess other industrially-important properties such as ethanol tolerance, thermotolerance and resistance to killer yeasts (Irene et al., 2009*). S. cerevisiae* F-727 was selected for further studies.

Effect of vinasse amount in fermentation medium

Vinasse replaced water in different levels for dilution molasses for obtaining desired sugars concentration in fermentation molasses medium (Table 2). Results show that up to 30% v/v replaced vinasse instead of water; there is no effect on ethanol yield or fermentation efficiency. There was slight efficiency when 40% v/v vinasse was used instead of water. There was relative decrease in ethanol yield and fermentation efficiency when vinasse was introduced above 50% v/v instead of water in the fermentation medium; but it increased as the level of vinasse increased. From the results obtained, it is suggested that vinasse should be increased instead of water in the fermentation medium and also to increase the inhibitors involved in the end fermentation whose vinasse is tacked from methanol, fusel alcohols, acetic acid, aldehides and aromatic compounds (Arshad et al. 2008). Heavy metals in the fermentation medium should be increased and as well as one or more minerals toxic to biomass production or their activity (Madaree et al., 1991). Other by-products of the metabolism of the yeast like glicerol, propanol, furfural and lactic acid also inhibit the fermentation and growth speed (Navarro et al., 2000). Besides the inhibitory effect of the fermentation by-products, the effect of the increased solids on the vinasse is very important, including the non-fermentable sugars remaining in the vinasse after fermentation and distillation. Mineral salts not assimilated by the yeast also increase with the increased vinasse fermentations, due to the molasses and the nutrients used for preparing the medium of fermentation (Maiorella et al., 1984).

Table 3. Effect of urea supplementation on the ethanol yield production from sugar cane molasses diluted with different amounts of vinasse instead of water by *S. cerevisiae* F-727.

Vinasse (%, v/v)	Urea concentration (ppm N/l)							
	-		100		200		300	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	11.0	100.0	11.42	100	11.46	100.0	11.34	100.0
10	11.0	100.0	11.42	100	11.46	100.0	1.341	100.0
20	11.1	100.1	11.46	100.4	11.46	100.0	11.28	99.5
30	11.0	100.0	11.38	99.7	11.36	99.1	1.261	99.4
40	10.9	99.9	11.22	98.3	11.12	97.4	11.10	97.9
50	10.5	98.2	11.06	96.9	11.10	97.2	10.94	96.5
60	10.6	96.4	10.72	93.9	10.76	94.2	10.68	94.2
70	10.3	93.7	10.40	91.1	10.22	89.5	10.42	91.9
80	9.9	90.0	10.08	88.3	10.09	88.4	9.98	88.0
90	9.6	87.3	9.77	85.6	9.84	86.2	9.68	85.4
100	9.5	86.4	9.40	82.3	9.46	82.8	9.50	83.8

EtOH, Ethanol yield; FE, fermentation efficiency.

Effect of nitrogen sources

Urea

Urea was used as cheap nitrogen source in producing alcoholic fermentation. Table 3 shows that it enhanced the ethanol yield as well as the fermentation efficiency (FE) compared to the medium free from nitrogen source. More enhancement was achieved when 150 ppm of urea was introduced in the molasses fermentation medium diluted with water and 100 ppm of urea in the molasses fermentation medium diluted with vinasse. From this, the yeast strain can utilize the residual nitrogen involved in vinasse. There is the possibility of saving an amount of nitrogen when vinasse is recycled as diluents in fermentation medium. Nitrogen deficiency slows down yeast growth and the fermentation efficiency (Dukes, 1991), due to the inhibition of the synthesis of protein transporting sugar through the cell membrane to the interior of the cells. It has been shown that an adequate nitrogen increases yeast growth provided the other essential yeast nutrients are not lacking (Nofemele et al., 2012). Guojun et al. (2012) concluded that production of high levels of ethanol could be achieved by supplementing urea as nitrogen source during ethanol fermentation.

Effect of diammonium phosphate (DAP)

Diammonium phosphate was used as good nitrogen and phosphorus sources in fermentation medium for ethanol yield (Arshad et al., 2008). Table 4 shows that adding 300 mg Nl^{-1} of DAP to the fermentation medium was more suitable than low or high concentrations. Arrizon and Gschaedle (2002) evaluated the effect of assimilable nitrogen on growth in batch cultures of *S. cerevisiae* under different nitrogen concentrations (from 16·5 to 805 mg N l^{-1}). They showed that lower than 66 mg N l^{-1} slowed down cell growth and a relevant decrease in cell biomass was observed. In the strain, it appears the highest biomass production was observed in the media with 402 mg N l^{-1}. The kinetics of glucose and nitrogen consumption indicates that the ability of the yeast strain to break down sugars is strongly connected with increased nitrogen availability in the media. The results show that supplementation with diammonium phosphate added during fermentation increased cell population, fermentation rate and ethanol yield. The ammonium ion also serves as an allosteric regulator for one of the enzymes used in glycolysis and may also have an effect on how the yeast cell transports glucose and fructose into the cell (Arrizon and Gschaedler, 2002).

Effect of ammonium sulfate

Fermentation rate and the time required for completion of the alcoholic fermentation are strongly dependent on nitrogen availability. At the stationary phase, the addition of nitrogen source is effective in increasing cell population, fermentation rate and ethanol yield (Ferreira-Mendes et al., 2004). The obtained data revealed that the addition of ammonium sulfate to fermentation medium at any level resulted in decrease in ethanol yield by *S. cerevisiae* F-727. Our finding agrees with previous studies which show that there were significant differences in the amount of ethanol produced when ammonium sulfate was added to the fermentation medium, although this depended on the yeast strain used. When assimilable nitrogen was added, ethanol production

Table 4. Effect of diammonium phosphate supplementation on the ethanol yield production from sugar cane molasses diluted with different amounts of vinasse instead of water by *S.cerevisiae* F-727.

Vinasse (%, v/v)	Diammonium phosphate concentration (ppm N/l)							
	-		100		150		200	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	11.0	100.0	11.62	100.0	11.82	100	11.80	99.2
10	11.1	100.0	11.62	100.0	11.82	100	1.701	99.2
20	11.1	100.1	11.72	100.7	11.82	100	11.70	98.6
30	11.0	100.0	11.82	101.7	11.62	98.3	1.641	95.8
40	10.9	99.91	11.82	101.7	11.52	97.5	11.30	95.3
50	10.8	98.23	11.62	100.0	11.44	96.8	11.24	93.2
60	10.6	96.42	11.44	98.5	11.29	95.2	11.00	91.7
70	10.3	93.71	11.12	95.7	11.02	93.2	10.82	86.8
80	9.9	90.00	10.82	93.1	10.88	92.1	10.24	85.3
90	9.6	87.32	10.27	88.4	10.26	86.8	10.06	83.6
100	9.5	9.96	9.96	85.7	9.96	84.3	9.86	100

EtOH, Ethanol yield; FE, fermentation efficiency.

Table 5. Effect of ammonium sulfate supplementation on the ethanol yield production from sugar cane molasses diluted with different amounts of vinasse instead of water by S. cerevisiae F-727.

Vinasse (%, v/v)	Diammonium phosphate Concentration (ppm N/l)							
	-		100		150		200	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	11.0	100.0	11.02	100	10.92	100	10.80	100
10	11.1	100.0	11.00	99.9	10.90	100	10.80	100
20	11.1	100.1	10.96	99.5	10.90	100	10.76	99.6
30	11.0	100.0	10.90	98.9	10.58	96.9	10.46	96.9
40	10.9	99.91	10.68	96.9	10.42	95.4	10.22	94.6
50	10.8	98.23	10.62	96.4	10.38	95.1	10.18	94.3
60	10.6	96.42	10.58	96.0	10.30	94.3	10.08	93.3
70	10.3	93.71	10.50	95.3	10.00	91.6	9.96	92.2
80	9.9	90.00	10.48	95.1	9.26	84.8	9.16	84.8
90	9.6	87.32	9.66	87.7	9.26	84.8	9.06	83.9
100	9.5	9.96	9.36	84.9	9.04	82.8	9.00	83.3

EtOH, Ethanol yield; FE, fermentation efficiency.

either increased (with *S. cerevisiae* AR2 and *S. cerevisiae* NT116 yeast strains) or decreased (with *S. cerevisiae* LW LVCB CT1+ yeast (Hernandez-Orte et al., 2006). Also, nitrogen added in the form of ammonium sulfate did not affect the ethanol production rate (Arshad et al., 2011).

Table 5 shows the effect of ammonium sulfate supplementation on the ethanol yield obtained from sugar cane molasses diluted with different amounts of vinasse instead of water by *S. cerevisiae* F-727.

Effect of orthophpsphoric acid (OPA) supplementation

Table 6 shows that the supplementation of OPA plus urea in fermentation medium enhanced ethanol yield and raised the possibility of applying vinasse in fermentation medium up to 40% instead of water. Data shows also that maximum ethanol yield can be obtained by using low amount of OPA in the case of fermentation molasses medium diluted with vinasse. Nitrogen and phosphorus are the main nutritional requirements for yeast growth and maximum ethanol production efficiency. Although molasses contains most of the nutrients required for yeast growth, generally nitrogen and phosphate are added to enhance yeast growth and ethanol production (Malherbe et al., 2007). For optimum yeast efficiency in molasses medium, OPA was used as phosphate source. Phosphorus plays the major role in the glycolysis cycle in yeast cell. Extensive studies were previously performed

Table 6. effect of orthophosphoric acid supplementation on the ethanol yield production from sugar cane molasses diluted with different amounts of vinasse instead of water by *S.cerevisiae* F-727.

Vinasse (%, v/v)	Orthophosphoric acid (ppm)							
	-		25		50		75	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	11.42	100.0	11.72	100.0	11.78	100	11.70	100
10	11.42	100.0	11.72	100.0	11.78	100	1.701	100
20	11.46	100.1	11.82	100.2	11.82	100.2	11.72	100.2
30	11.38	100.0	11.70	99.9	11.72	99.5	1.601	99.2
40	11.22	99.91	11.46	97.8	11.52	97.8	11.36	97.1
50	11.06	98.23	11.22	95.7	11.12	94.4	11.24	96.1
60	10.72	96.42	11.14	95.1	11.04	93.7	11.00	94.0
70	10.40	93.71	10.70	91.3	10.72	91.0	10.92	93.3
80	10.08	90.00	10.32	88.1	10.18	86.9	10.54	90.1
90	9.77	87.32	9.96	85.0	9.96	84.6	10.26	87.7
100	9.40	9.96	9.72	82.9	9.76	82.9	9.86	84.3

EtOH, Ethanol yield; FE, fermentation efficiency.

Table 7. Effect of magnesium sulfate supplementation on the ethanol yield production from sugar cane molasses diluted with different amounts of vinasse instead of water by *S. cerevisiae* F-727.

Vinasse (%, v/v)	Magnesium sulfate concentration (ppm)							
	-		15		30		45	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	11.72	100.0	11.88	100	12.00	100	12.00	100
10	11.78	100.1	11. 88	100.1	12.00	100	12.00	100
20	11.82	100.1	11.94	100.1	12.08	100	12.O0	100
30	11.70	100.0	12.00	100.1	12.12	100	12.08	98.5
40	11.46	99.91	11.82	100.2	12.00	100	11.90	97.8
50	11.22	98.23	11.22	100	11.34	99.2	11.32	94.6
60	11.14	96.42	11.04	99.6	11.40	98.8	11.04	93.1
70	10.60	93.71	10.80	97.2	11.62	98.2	10.70	90.2
80	10.32	90.00	10.42	92.3	10.46	95.3	10.44	88.0
90	9.96	87.32	9.86	89.8	9.36	92.4	10.16	85.7
100	9.42	86.41	9.22	87.1	9.36	90.1	9.22	77.7

EtOH, Ethanol yield; FE, fermentation efficiency.

to optimize nitrogen and phosphorous sources and other supplements (Pretorius et al., 2013). Higher ethanol production has also been previously reported with urea and phosphoric acid, making the process very economical (Arshad et al., 2008).

Effect of varying concentrations of magnesium sulfate

Varying concentrations of magnesium sulfate were supplemented in fermentation medium diluted with different percents of vinasse compared to those diluted with fresh water under the above optimized nutrients levels of urea and orthophosphoric acid (Table 7). Results show that ethanol yield as well as relative fermentation efficiency was enhanced by introducing magnesium sulfate in molasses medium diluted with vinasse, especially in the medium diluted with vinasse above 40%. The data shows that the demand level of magnesium sulfate was increased in the medium diluted with vinasse than that diluted with water; this led to maximum ethanol production. Also, the addition of magnesium enabled yeast strain to utilize some fermentable sugars involved in vinasse to yield more ethanol than that diluted with fresh water. The data agree

Table 8. Effect of varying inoculum size on ethanol yield from sugar cane molasses diluted with different amounts of vinasse instead of water by *S.cerevisiae* F-727.

Vinasse (%, v/v)	Inoculum size (%, v/v)							
	10		15		20		25	
	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)	EtOH (%)	FE (%)
0	12.00	100	12.00	100	12.00	100.0	11.98	100.0
10	12.00	100	12.00	100	12.00	100.0	11.98	100.0
20	12.08	100	12.00	100	12.00	100.0	11.98	100.0
30	12.12	100	12.08	100.7	12.08	100.7	12.18	101.7
40	12.00	97.8	12.22	101.8	12.28	102.3	12.32	102.8
50	1174	96.7	12.o2	100.2	12.12	101.0	12.22	102.0
60	11.60	95.0	11.76	98.00	11.81	98.4	11.78.	98.3
70	10.40	85.9	11.64	97.00	11.62	96.8	11.60	96.8
80	10.26	82.2	10.70	89.20	10.96	91.3	11.00	91.8
90	9.86	80.5	10.24	85.30	10.52	87.7	10.65	88.9
100	9.66	100	9.72	81.00	9.84	82.0	9.90	82.6

EtOH, Ethanol yield; FE, fermentation efficiency.

with scientific knowledge that vinasse contains high level of potassium which inhibits the metabolism of magnesium. As a result, more magnesium is needed to overcome this problem. Deficiencies and imbalances in minerals and cations serving as co-factors for glycolytic and other enzymatic reactions can result in fermentation arrest (Blackwell et al., 1997). Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway and subsequently ethanol production (Walker, 1994).

Effect of varying inoculum sizes

Ethanol yield and production of co-products has a major relationship in ethanol fermentation. Extensive studies have been carried out to investigate the effect of yeast inoculation rate to help the yeast cells overcome the bacterial cells on the basis of size and number (Fadel et al., 2013). Effect of varying inoculum sizes on ethanol yield was studied under the above optimized parameters. Maximum ethanol content was found when the inoculation rate was 25% v/v and was 12.3% v/v in fermentation medium diluted with 50% vinasse instead of water (Table 8). The obtained results showed that yeast cells absorb heavy metals which inhibit the enzymatic system responsible for ethanol production by yeast cells, and as the number of cells increases the amount of heavy metals per cell decreases from the surrounding medium. In brewing, higher yeast inoculum causes attenuation to initiate the process more rapidly, and reduces viability losses that occur immediately after pitching. In a previous study, ethanol yield increased with increasing inoculum size and yield of methanol, and aldehydes were the lowest when inoculum size was above 30% (Arshad et al., 2008).

Effect of dilution with vinasse on fermentation time course

Table 9 shows that the fermentation medium diluted with above 30% (v/v) increased the fermentation time, leading to the production of the highest ethanol in the fermentation mash. It can be said that the inhibitory effect of heavy meals (Arshad et al., 2008) as well as osmoses effect increased with the amount of vinasse added to the fermentation medium (Patrascu et al., 2009).

Industrial application

Vinasse [25 % (v/v)] was applied instead of water in diluted fermentation medium as well as 100% (v/v) in industrial fermenters of 65 m^3 capacity. Table 10 shows the safe means of using vinasse obtained from the fermentation of sugar cane molasses for ethanol production in distillery factories without affecting both ethanol yield and fermentation efficiency (FE). Also, the yeast can partially utilize some residual fermented sugars involved in vinasse, and this has the advantage of raising the distillery factory efficiency.

Conclusion

This study showed the safe possibility of using up to 50 % vinasse resulting from the fermentation of sugar cane molasses for ethanol production instead of fresh water in distillery factories without affecting both ethanol yield and fermentation efficiency as well as the yeast can partially utilized some of residual fermented sugars and nutrients involved in vinasse and this has advantage in rising the distillery factory efficiency causing low ethanol cost production. On the other hand aids in the solution of the environmental problem for vinasse disposal.

Table 9. effect of dilution with vinasse on fermentation time course on ethanol yield from sugar cane molasses diluted with different amounts of vinasse instead of water by *S.cerevisiae* F-727.

Vinasse (%, v/v)	Fermentation time (h)							
	24		30		36		42	
	EtOH (%)	RS (%)	EtOH (%)	RS (%)	EtOH (%)	RS (%)	EtOH (%)	RS (%)
0	11.20	3.30	11.88	1.74	11.98	1.64	11.94	
10	11.20	3.31	11.88	1.75	11.98	1.64	11.94	1.64
20	11.16	3.77	11.90	1.76	11.98	1.76	11.98	1.75
30	11.04	4.37	12.08	1.86	12.18	1.96	12.28	1.76
40	11.02	4.40	12.32	2.10	12.32	2.10	12.36	186
50	11.02	4.41	12.02	2.82	12.22	2.68	12.24	2.06
60	10.90	4.64	11.32	3.36	11.78	3.26	11.86	2.64
70	10.74	5.20	11.08	4.64	11.60	4.64	11.60	3.16
80	10.60	5.48	10.20	5.44	11.00	5.44	11.0	4.64
90	10.22	6.28	10.44	6.12	10.65	5.92	10.65	5.44
100	9.72	**7.08**	9.90	7.00	9.94	6.98	9.98	5.92

EtOH, Ethanol yield; FE, fermentation efficiency.

Table 10. Effect of dilution with vinasse instead of water on ethanol yield and fermentation efficiency from sugar cane molasses by S. cerevisiae F-727 .in Hawamdia Distillation Factories.

Vinasse (%)	TFS (%)	pH	EtOH (%)	RS (%)	FE (%)
0	17.22	4.85	9.10	2.44	87.6
5	17.24	4.85	9.10	2.22	87.6
10	17.28	4.76	9.12	2.46	87.6
15	17.36	4.78	9.15	2.52	87.5
25	17.48	4.89	9.20	2.76	87.2
100	17.86	4.90	7.90	5.64	73.7

Total fermented sugars (TFS); EtOH, Ethanol yield; RS, residual sugars; FE, fermentation efficiency.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

The role of bone marrow derived mesenchymal stem cells in induced stroke

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Stroke is the third most common cause of death, and a leading cause of physical disability in adults. Recovery after a major stroke is usually limited, but cell therapy, especially by application of mesenchymal stem cells (MSCs) is emerging with fixed neurologic deficits. The aim of the current study was directed to isolation and cultivation of the bone marrow (BM) derived MSCs from young rats, as well as to study the role of intravenous administration of BM-MSCs in mature male rats as an animal model for Middle Cerebral Artery Occlusion (MCAO). MSCs are spindle in shape fibroblast-like cells and possess the ability to aggregate and form colonies-forming unit – fibroblast (CFU-F). MSCs showed positive response for $CD105^+$ (the specific marker for MSCs detection) and negative response for surface marker ($CD34^-$), characteristic for the hematopoietic cells. The immunohistochemistry study of intravenous administration of Bromodeoxyyuridin (BrdU) labeled BM-MSCs after 24 h of mechanical MCAO in mature rats, demonstrated survival, engraftment and migration of systemically delivered cells in the cerebral cortex and heart tissues. However, these cells were not indicated in the lung and liver tissues. In conclusion, intravenously administered BM-MSCs enter brain and heart, and survive of this, may provide a cell source to treat stroke and heart disease.

Key words: Middle cerebral artery occlusion, mesenchymal stem cells, rats, transplantation, bromodeoxyyuridin.

INTRODUCTION

Bone marrow derived mesenchymal stem cells (BM-MSCs), like other stem cells, have the capacity of unlimited self-renewal and they give rise to differentiated cells from various cell lineages (Doeppner and Hermann, 2010). This means, MSCs is not only differentiated into types of cells of mesodermal lineage, but also into endodermal and ectodermal lineages. MSCs have been identified as an adherent, fibroblast-like population, and

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Abbreviations: MSCs, Mesenchymal stem cells; **BM,** bone marrow.

they can be isolated from different adult tissues, including BM, umbilical cord, skeletal muscles and adipose tissue (Li and Ikehara, 2013). There are some characteristics that make MSCs safe and promising candidates for application in tissue regeneration procedures. Progenitor and pluripotent MSCs, which are located in all tissues and organs, have a connective tissue component, with the potential to form at least four tissues of mesodermal origin (Young et al., 1995), for instance MSCs have been purified from articular cartilage culture (Eslaminejad and Taghiyar, 2007). Beside, broad range of MSCs distribution, these cells are free from ethical concerns, they also possess non-immunogenic properties, have injury-seeking capabilities, and can be used as vehicles for gene therapy (Zomorodian and Eslaminejad, 2012). Accumulating evidences support the beneficial effects of MSCs transplantation: in neurodegenerative diseases (Torrente and Polli, 2008); for attenuation pain, induced by spinal cord injuries (Takikawa et al., 2013); in both patients with Ischemic Stroke (Lee et al., 2010) and animal models of stroke (Chen et al., 2001b; Doeppner and Hermann, 2010).

Stroke is a leading cause of death, along with cancer and coronary heart disease, and it is the most common cause of physical disability in adults (Kim et al., 2013). Approximately 80% of all strokes are ischemic with occlusion of a cerebral artery, leading to infarction of brain tissue and consequent death of neurons and/or glia. Subsequent symptoms depend on both the location of the lesion and the cell types lost (Williamson et al., 2008). Moreover, stroke causes a greater loss of healthy life years, as measured in disability-adjusted life years, than over illnesses (Hong et al., 2011). Recently, cell-based therapy has been evaluated as a regenerative strategy for patients with fixed neurologic deficits after stroke (Kim et al., 2013). Therefore, several clinical trials have used BM-MSCs in stroke and the results from these studies have raised important issues. Specifically, different variations according the patient characteristics, cell therapy timing, dose and type of cells delivered and mode of treatment, have been noted (Bhasin et al., 2011; Savitz et al., 2011; Friedrich et al., 2012; Kim et al., 2013).

Since the BM is very an important source of adult stem cells, especially of MSCs, the aim of the current study was connected with isolation and cultivation of BM-MSCs from young rats, to maintain them through several passages, and to study the role of intravenous administration of BM-MSCs, derived from different passages in stroke.

MATERIALS AND METHODS

Isolation and cultivation of BM-MSCs

Mesenchymal stem cells (MSCs) were harvested from young albino male rats (53 to 58 days old and weighting 128 to 197 gm). Rats were obtained from the Animal Breeding House, College of Education, University of Duhok, Duhok, Iraq. The animal experiments were done in Animal Tissue Culture Laboratory, Scientific Research Center, University of Duhok. The rats were sacrificed by using chloroform, and the BM-MSCs cultures were prepared according to Peister et al. (2004) with some modification. Briefly, under sterile conditions, BM was harvested by flushing the tibial and femoral bone marrow cavities with complete culture media [Iscove's modified Dulbecco medium (IMDM) (US Biol-USA), supplemented with 15% fetal bovine serum (FBS) (Invitrogen)]. Our modification was the use of IMDM instead of RPMI-1640 medium, and 15% FBS instead of 9% FBS and 9% horse serum which were used by Peister et al. (2004). Marrow plug suspension was dispersed by Pasture pipette. The so-called obtained cells suspension was resuspended in 4 to 5 ml of phosphate buffer saline (PBS), and layered over an equal volume of Ficoll-Paque (1.077 g/ml) (US Biol –USA). Then, the cell suspension was centrifuged at 2500 to 3000 rpm for 25 min at 8°C. After density gradient centrifugation, the resulting mononuclear cells (MNCs) and stem cells populations were retrieved from the Buffy coat and placed in a sterile conical tube, then washed two times with PBS and centrifuged at 2000 rpm for 10 min.

The MNCs derived stem cells population suspension were seeded in 25 cm^3 plastic tissue culture flasks (Denemark: Nunc) with 5 ml IMDM plus 15% FBS, and cultivated in incubator with 5% CO_2 (Korea LabTecl) at 37°C in a humidified atmosphere for 21 days, until they reached confluence and were defined as passage 0. Cultures of MSCs were inspected daily and re-fed every three days by replacing half of culture medium by an equal volume of fresh medium. The MSCs were isolated on the basis of their morphology and ability to adhere to the tissue culture flask (Javazon et al., 2001). So, when the culture reached approximately 80 to 90% monolayer confluence, the cells were recovered using 0.25% trypsin-EDTA and the final product was resuspended in 1 ml of IMDM plus 15% FBS. Then, the cell number and viability were determined using 0.4% trypan blue prepared in 0.9% normal saline (Buzanska et al., 2002). The cells were re-cultured in new plastic tissue culture flasks at a density of 5 X 10^4 cells / cm^2.

Immunophenotypic analysis of BM-MSCs

At the 2nd passage, BM-MSCs were trypsinized into single cell suspension and re-cultured in multi-well tissue culture plates (4-wells), containing Poly-L-Lysine (Sigma-Germany), pre-coated cover slides at a density of 1×10^3 cell/well in IMDM medium, supplemented with 15% FBS. The cultures were maintained in CO_2-incubator, in the same culture conditions until they reached confluence. The attached cells were washed with PBS and fixed with 4% Phosphate buffered formalin for 10 min, then detected by immunocytochemistry method using mouse monoclonal antibodies against human CD105 and CD34 (Abcam). This procedure was performed according to the manufacturer's instructions of these CD markers (Buzanska et al., 2002) and counter stained with Harris Hematoxylin.

Stroke induction

Animal model for middle cerebral artery occlusion (MCAO)

Adult albino male rats (n=28), weighing 270 to 300 g, were subjected to middle cerebral artery occlusion (MCAO). The animals were anaesthetized with 70 mg/kg body weight intra-peritoneal (i.p.) injection of ketamine and 5 mg/kg body weight xylazine (Gonzalez and Klob, 2003). MCAO was induced by a method of intra-luminal vascular occlusion described by (Chen et al., 1992; 2001a). Briefly, 1.5 cm incision was made in the right side of the animal neck, and then sterile small size cannula was gently inserted into the right common carotid artery and advanced toward the internal carotid

Figure 1. A. The right common and external carotid arteries were expressed carefully through incision. **B.** Insertion of the needle into the right common carotid artery gently. **C.** Insertion of nylon suture (blue colour) through the cannula to reach the origin of the MCA after withdrawal. **D.** The wound sutured by catgut suture after withdrawal of the nylon suture and the cannula. **E.** Intravenous administration of Brdu labeled BM-MSCs (through the tail vein) via 27 gauge needle slowly.

artery. After withdrawal of the needle, a length of 4.0 monofilament nylon suture (18.5 to 19.5 mm), determined by the animal weight, was inserted into the cannula and advanced toward the origin of the middle cerebral artery (MCA). Three hours after MCAO, reperfusion was performed by withdrawal of the cannula and nylon suture, the wound was sutured by catgut (Figure 1A, B, C and D). The rest of the body placed over thermo regulated operating table, fixed at 37°C.

MCAO animals groups

MCAO animals groups (n=28) were divided into four groups (n=7 for each group), as follows: groups (1, 2, 3): rats of these groups were injected intravenously after 24 h of stroke induction with MSCs at 2nd, 8th and 12th passages, respectively. While, group (4) (first control group) were rats given MCAO alone without donor cell administration. In addition to these groups, this experiment also included second control group (5) (n=7), but these were rats without given MCAO and cell administration, that means normal rats without any treatment.

Labeling of mesenchymal stem cells

In order to study the capability of rat BM-MSCs to maintain their plasticity in different passages for survival, multiplication and migration after intravenously administration in rat animal models for MCAO, the 2nd, 8th and 12th passages of BM-MSCs were maintained to grow in culture. When the culture reached approximately 80% monolayer confluence, the culture medium was removed and replaced with fresh culture medium (IMDM plus 15% FBS), containing 4 µg/ml Bromodeoxyuridin (BrdU) (Sigma, Germany), to label MSCs for intravenous administration in rat model of stroke (Seghatoleslam et al., 2012).

Intravenous administration of BrdU-labeled mesenchymal stem cells

The BM-MSCs were maintained with labeling medium (IMDM plus 15% FBS and 4 µg /ml BrdU) for 72 h, then this medium was aspirated, the cells were washed with PBS and trypsinized into single cell suspension. Then, for each animal from groups 1, 2 and 3, after 24 h of stroke induction, 27 gauge needle loaded with the 1X10^{6} BrdU- labeled MSCs in 500 µl culture medium were slowly injected intravenously into the tail vein within 2 to 3 min (Figure 1E). All transplantation procedures were performed under aseptic conditions and immuno-suppressors were not used in any experimental animal.

Histological study

Animals of groups 1, 2 and 3 were allowed to survive for 31 days after MSCs transplantation, after which these animals were sacrificed by chloroform, whereas the animals from the first control group (4) were sacrificed after 24 h of stroke induction (to show the eventual changes in the brain at this time point). Animals from the second control group (5) were also sacrificed by chloroform. Generally, the skin, muscles and skull bones were dissected out with suitable surgical instruments, and then the whole brain was manipulated carefully and fixed in 10% Phosphate buffered formalin at room temperature. In parallel, the animals' hearts, livers and

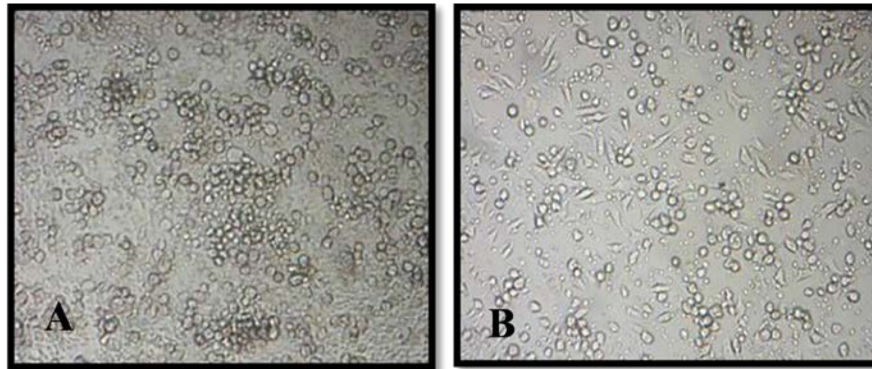

Figure 2. Primary culture of young rat BM under inverted microscope (X100). **A.** The cells after 2 to 3 days, most of BMCs are floated and the remaining of cells began to attach. **B.** after 4 to 5 days some of cells became elongated and have a fibroblast-like morphology and other cells appear as small round cells.

lungs were removed and also fixed by 10% Phosphate buffered formalin at room temperature. These organs were embedded in paraffin, in order to be ready for immunohistochemical assay. The BrdU-labeled cells were detected in sections according to manufacturer's instructions of the markers by light and fluorescent microscope.

RESULTS

BM-MSCs culture and detection

The first step in this study was connected with laboratory cultivation of BM-MSCs, obtained from young rats. The results show that in the initial BM-MSCs culture, two major types of cells were observed; hematopoietic stem cells (HSCs) and MSCs. During first hours of culturing, these cells were floating in culture medium then; some of them began to adhere progressively to the tissue culture flask surface. After two to three days of cultivation, these cells became elongated and received small uni-polar processes or fibroblast like-spindle shape (Figure 2A and B). While, the non-adherent cells (HSCs) were removed and discarded through the continuous medium changing, by replacing half of culture medium with an equal volume of fresh medium. As a result of continuous medium changing, it became clear to distinguish adherent cells with proliferation activity. The most characteristic *in vitro* feature of MSCs was their ability to aggregate and form colonies, dispensed in the culture. After 9 to 10 days from the initial cultivation, numerous colonies of different sizes appeared in the culture, each colony was derived from single adherent cells and termed as a colony forming unite–fibroblast (CFU-F) (Figure 3A). Within time of culture, cells' proliferation resulted in forming population of fibroblast-like cells, which were shown around these colonies; such cells tend to connect the adjacent colonies (Figure 3B and C). After 21 days, the adherent cells reached approximately 80 to 90% confluence and formed homogenous monolayer of spindle-like cells, which is the typical shape of the MSCs (Figure 4A). These adherent cells (passage 0) were washed and harvested. The harvested cells from primary culture of rat BM were re-cultivated at ratio of 1:3 into new flasks and incubated in the culture medium, described above. This new culture has constituted the cells of first passage. Within 2 to 3 days, the so re-cultivated cells were observed retaining their morphology and become elongated, spindle in shape, like fibroblasts. In addition, these cells were maintained in their ability to generate CFU-F. The so formed colonies were increased in size and formed homogenous mono-layers of MSCs. Gradually, the monolayer of BM-MSCs tend to appear as homogenous layer (the majority of cells were spindle–like), this aspect for instance, is represented by MSCs at different passages. The MSCs were generally expanded in culture for twelve passages, remaining undifferentiated.

The majority MSCs of the 2nd passage (Figure 4B), indicated positive response for $CD105^+$, the specific marker for MSCs detection, and they appeared in green color of fluorochrome-conjugated secondary antibody (Figure 5A, B and C). However, these cells were negative for surface marker ($CD34^-$), which is associated with HSCs, and they stained in blue color of counter stain Harris hematoxylin (Figure 5D). These results indicate that the so derived cells are MSCs, but not hematopoietic in origin.

MCAO in mature rats

MCAO was performed for the first time in Iraq, as described previously, and followed by reperfusion. To quantitate the changes that occur, the brains from animals of group 4 were examined in details after 24 h of MCAO. The gross anatomy of the rats from this group (4) revealed congestion, which was observed on the surface

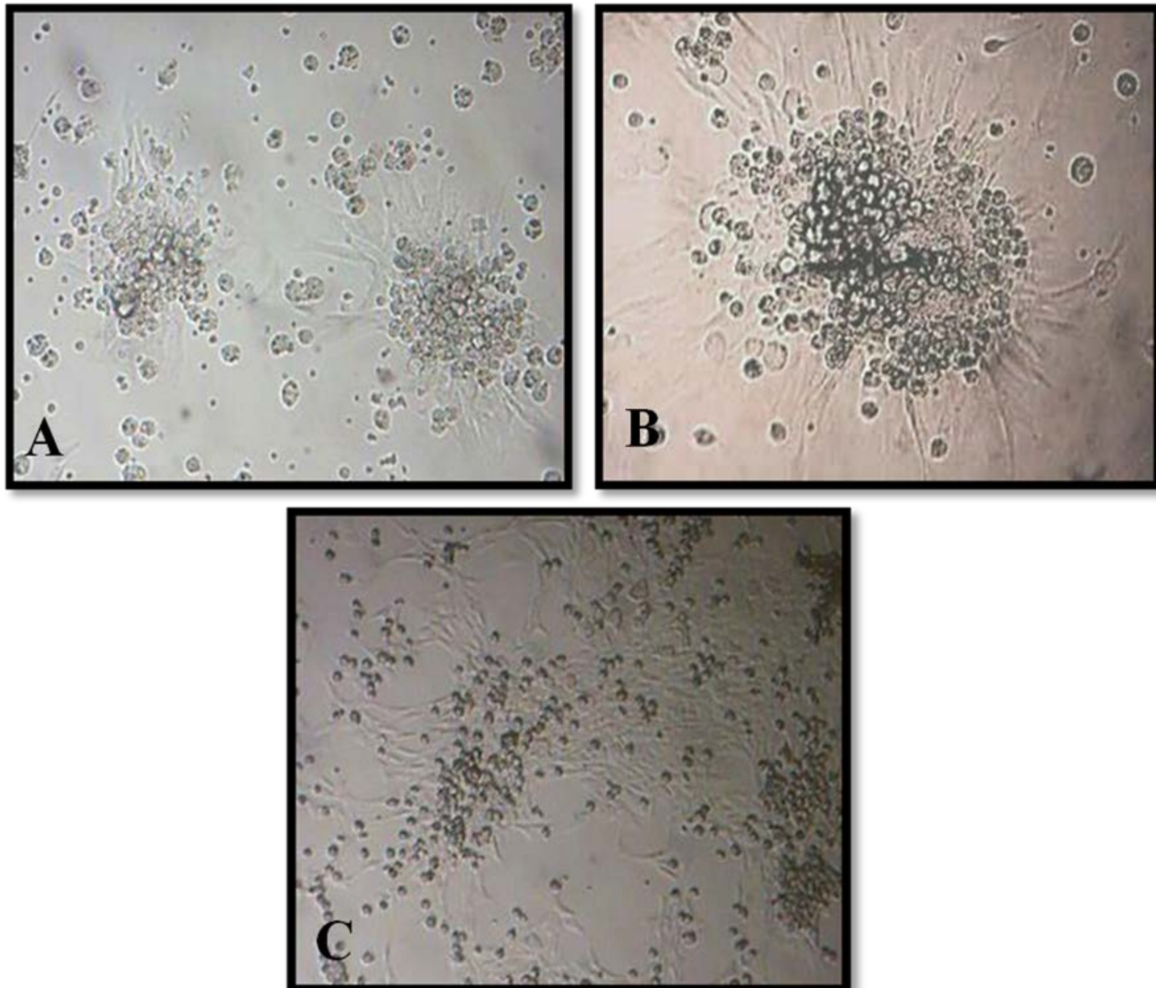

Figure 3. Illustration of the primary culture of BM-MSCs under inverted microscope. **A.** The formation of MSCs colony forming unit-derived fibroblast (CFU-F) (X100). B (X150) and C (X50): These figures show expanded MSCs colonies with numerous cells displaying fibroblast-like morphology.

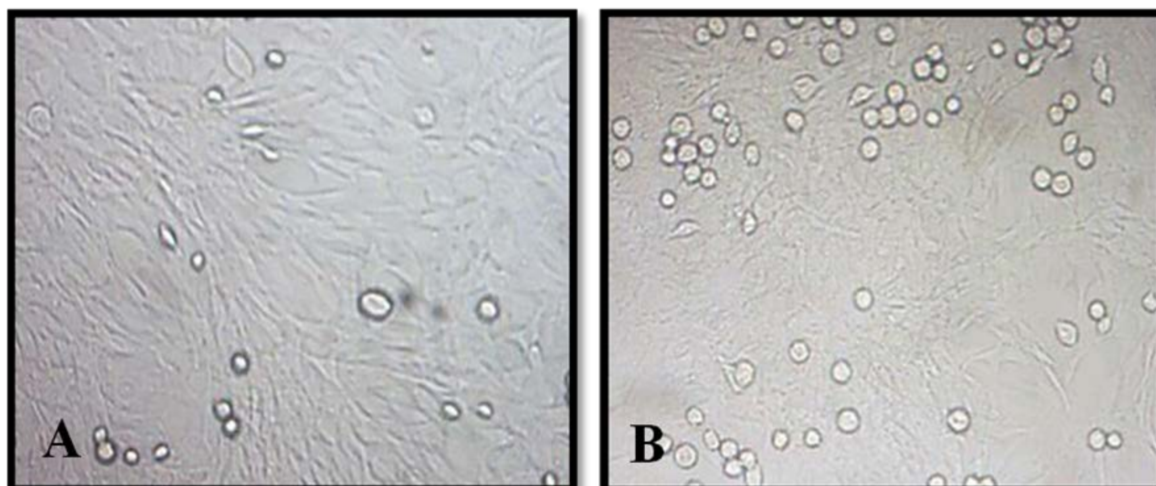

Figure 4. A, after 21 days from initial culture and B (2nd passages): The formation of homogenous layer of fibroblast-like cells with small round cells (X100).

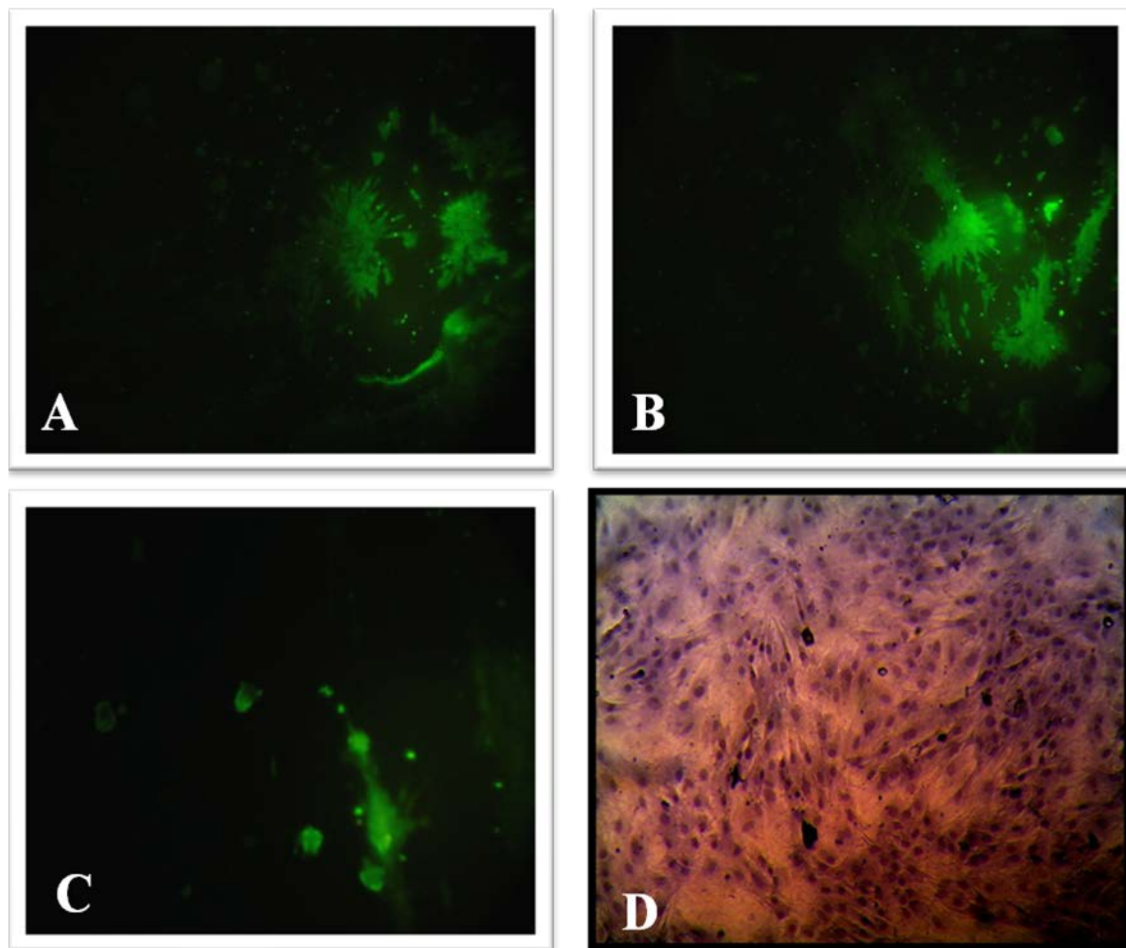

Figure 5. Immunophenotypic analysis of BM-MSCs *in vitro* at 2nd passage. **A, B** (X200), and C (X150): MSCs show positive response for CD105 (the specific marker for MSCs detection); cells revealed the colour of fluorescent conjugated secondary antibody (green colour). **D.** MSCs show negative response for CD 34 (the specific marker for Hematopoeitic stem cells detection), and stain with blue color (Hematoxylin stain) (X 100).

of the brain (Figure 6A). The congestion was noticeable in the middle cerebral artery MCA (Figure 6C). This effect of stroke induction in the experimental animals became clear in comparison with the brain of normal animals (group 5) (Figure 6B and D). Later, the experimental rats were inspected in their home cage environment, and no obvious movement abnormalities such as rotation or alteration of stereotyped behaviors were detected, that would distinguish them from controls. However, typical movements, associated with exploratory behavior, such as brief stopping, sniffing, rearing, or leaning against the cage wall were all reduced in the animals subjected to MCAO.

Histological and immunohistochemical studies

Immunohistchemical studies by light and fluorescent microscope were selected for demonstration of BM-MSCs engraftment, migration and long-term survival in the brain and other organs. Animals were allowed to survive for 31 days after intravenous administration of BM-MSCs; the examination of brain cross-sections, revealed the presence of BrdU-labeled cells as clusters. The migratory BrdU–labeled cells were located in different regions of the cerebral hemisphere, and specifically in the cerebral cortex (Figure 7A, B, C, and D). Despite the experimental groups, the microscopic examinations revealed the same ability of MSCs at different passages for migration and localization in the brain and heart tissues (Figure 8A and B). However, the investigations of liver and lung tissues revealed no BrdU-labeled cells, in addition we did not record any histo-pathological changes during examination of these tissues (Figure 8C and D).

DISCUSSION

In the present study, the culture of young male rats' BM-

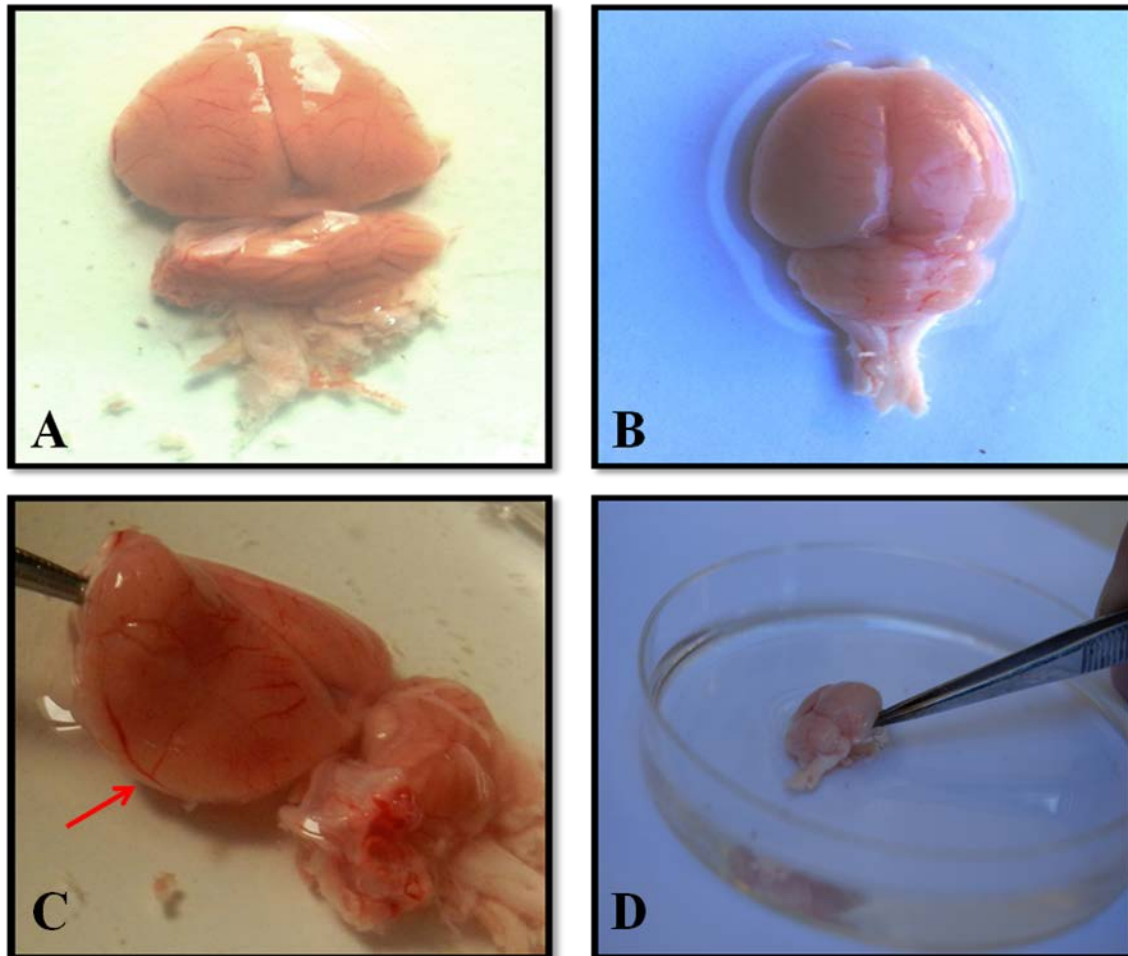

Figure 6. Photographs elucidate aspects of brain after 24 h of stroke induction (A, C left side) and show the comparison with the control brain (B, D right side), **A.** Congested blood vessels on the surface of cerebral hemispheres, **B.** The brain of control did not display any sign of congestion. **C.** The congestion of MCA (arrow) was quite obviously. **D.** In contrast the controls̕ cerebral hemisphere did not display congestion.

MNCs and stem cells populations resulted in appearance of a population of cells with fibroblastic morphology. These cells could be considered as MSCs, as described by many researchers, such as Friedenstein et al. (1970); Pittenger et al. (1999); Javazon et al. (2001); Eslaminejad et al. (2008) and Li and Ikehara, (2013). According to Friedenstein et al. (1970) and Javazon et al. (2001), MSCs in the present study were isolated on the basis of their morphology and ability to adhere on the tissue culture flask. One of the characteristic *in vitro*-features of MSCs is their ability to generate single cell-derived colonies of adherent cells. This single precursors cells with colony–forming ability are termed CFU-F, and they are usually used as an indicator for mesenchymal progenitor potential (Bochev et al., 2008). This feature of MSCs to generate CFU-F was observed in the present study, so many CFU-F with different sizes were observed that represent varying growth rates from cells with fibroblast-like spindle shape, migrating from these colonies.

The results of the immuno-phenotypic assay of BM-MSCs indicated that the majority of adherent cells and their colonies (CFU-F) were strongly stained by CD105. As indicated by Dominici et al. (2006), this molecule is an important CD marker, known to be expressed by MSCs. However, these cells did not express CD34, which is the specific marker, used for HSCs detection (Vogel et al., 2003). In order to facilitate a more purified approach for studying MSCs biology, the international for cellular therapy proposed minimal criteria to define MSCs as follow: 1. MSCs must be plastic–adherent. 2. MSCs must express CD105, CD73, and CD90 and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79 and/or HLA-DR surface molecules and 3. MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006).

However, Alt et al. (2011) had reported that conventional stem cell properties such as plastic adherence and

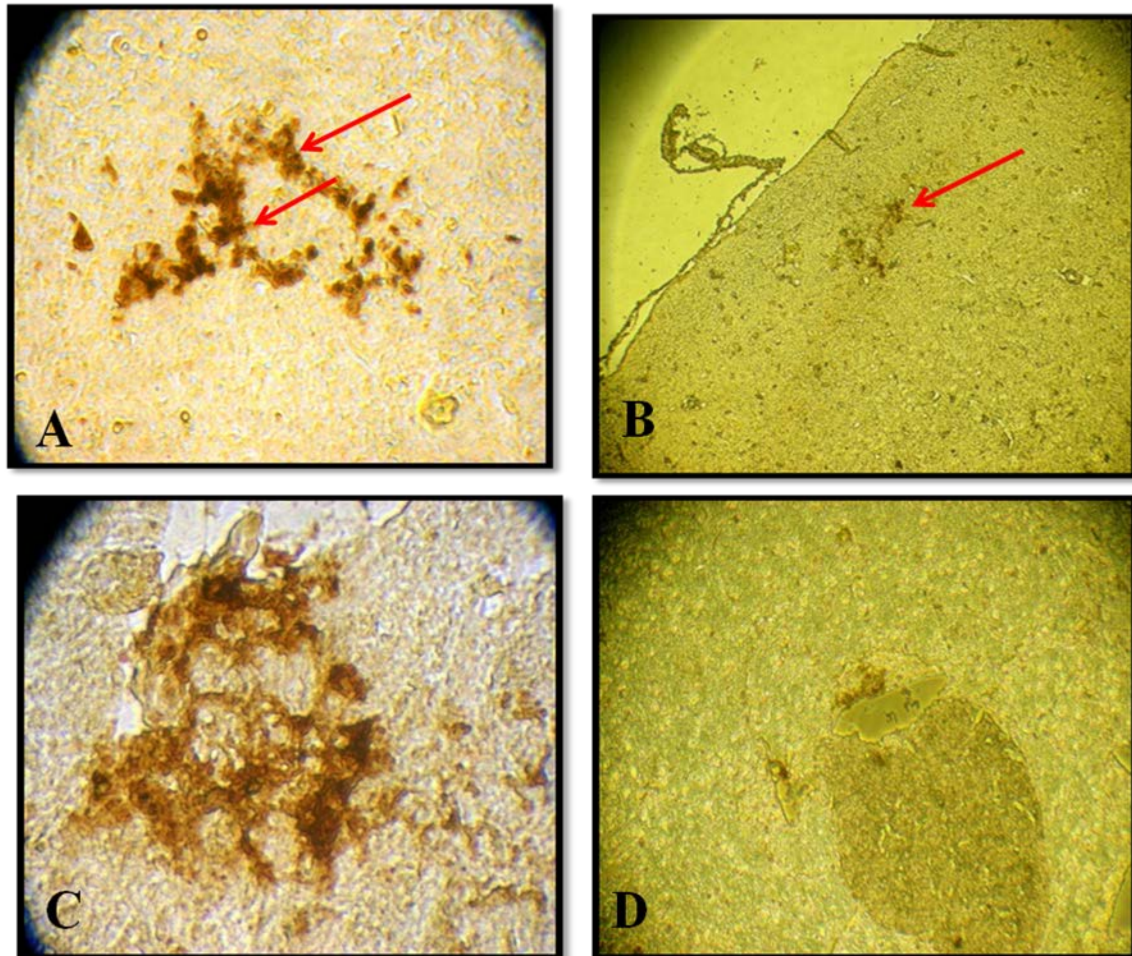

Figure 7. Photomicrographs of immunohistochemistry studies of the brain to determine the fate of BrdU-labeled BM-derived MSCs, after 30 day of cells infusion. A: BrdU –labeled MSCs appear as a clusters of cells in the brain, showing the intensity of staining after one step antigen retrieval (X200). B. Survival cells in the cortex of cerebral hemisphere injected by passage eight MSCs (X50). C. The intensity of staining reduced in cells migrated from cluster, may be this is attributed to clonal expansion (X400). D. Localization of BrdU –labeled cells in another region of the brain (X100).

the expression of CD105, CD44 and CD90 are unspecific for stem cells, unlike colony-forming capacity and differentiation capacity, which are specific properties, which differ MSCs from fibroblast.

MCAO in mature rats

Human specific disease is often random, sporadic and variable in its occurrence, and beyond a certain point, its biochemical and molecular complexities are simply inaccessible without turning to animal models, which offer reproducibility, replicability and control of confounding variable factors, essential to scientific hypothesis testing (Ginsberg, 2003). Since cerebral infarction is a heterogenous clinical entity with a variety of reasons such as etiology, localization, severity of ischemia and co-existing systemic diseases determining the outcome, these factors make clinical stroke characterization in human challenging. Many of these variables can be eliminated by employing an appropriate acute ischemic stroke (AIS), ischemic model enabling neuroscientists to focus on fundamental questions (Mehra et al., 2012). Transient occlusion of MCA by insertion of nylon suture in the right common carotid artery was preferred in this study, because this method is less invasive, cerebral artery occlusion produced is highly reproducible, and it is reversible allowing study of tissue reperfusion. This is in contrast to other methods like devascularization, which has limitations, although pia-stripping could induce cortical infarction, but mechanical damage to the underlying tissue and hemorrhagic does not permit reperfusion (Gonzalez and Kolb, 2003).

Histological and immunohistochemical assays

The immunohistochemical assays showed that after 31

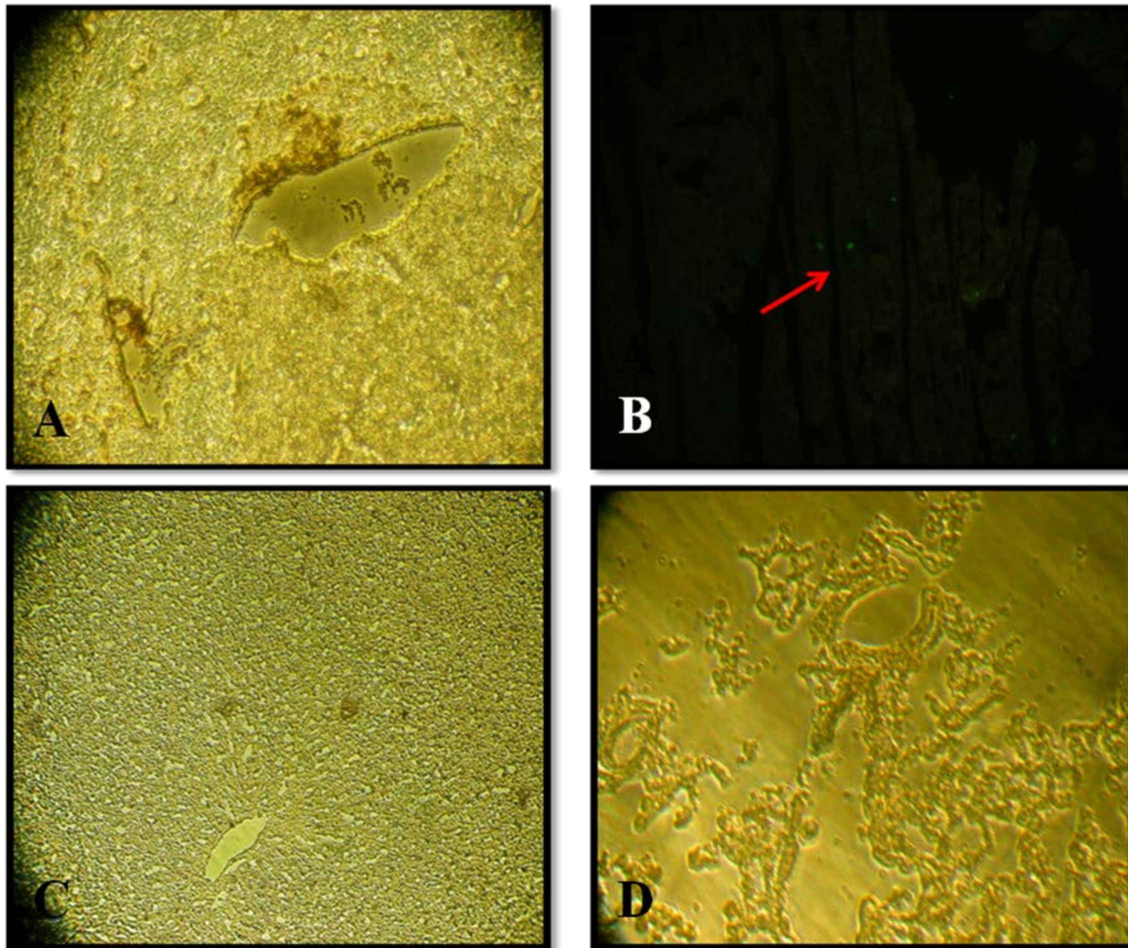

Figure 8. Microscopic examination of brain and other tissues in recipient rat of BrdU labeled cells. **A.** a number of cells reside around the blood vessel (X200). **B.** the micrograph demonstrate that some of the labeled cells reside in the heart (X100). **C.** this section of liver tissue was free from labeling cells (X100), D. Section in lung tissue of recipient rat, revealed no pathological changes that occur by the way as a result of systematic delivery of BM-derived MSCs (X200).

days from intravenous injection of BrdU, labeled MSCs at 24 h after stroke, these cells are more likely to enter into damaged brain and heart than into other organs such as liver and lung. This result was also reported by Chen et al. (2001a), but these authors demonstrated that intravenously injection of BrdU-labeled MSCs at 1 or 7 days after stroke significantly improved functional outcome, compared with non-treated rats. The most important interesting point in the present study is that the distribution of the labeled cells was not homogeneous. There were clusters of cells, which means that single progenitor cells underwent clonal expansion. This finding is in parallel with the study of Mezey et al. (2003), who observed this pattern of cells distribution during examination post-mortem brain samples from patients with lymphocytic leukemia, who had received bone marrow transplants. In this study, intravenous administration of BM-MSCs on the 2nd, 8th and 12th passages, offers an opportunity to highlight on some of MSCs characteristics, as follows: Despite the systemic delivery of BM-derived MSCs, these cells have been distinguished in the cerebral hemisphere, particularly in the cortex region. This can be attributed to the fact, that the MSCs possess the unique capacity to migrate or dock preferential to injured sites, due to expression of growth factors, chemokins and extracellular matrix receptors on the surface of MSCs (Meirelles et al., 2009; Rastegar et al., 2010).

In order to study the capability of rat BM-MSCs to maintain their survival, multiplication and migration on different passages, cells on the 2nd, 8th and 12th passages were chosen, to ensure that the delivered cells are nearly homogenous population, and hence no great difference between their migration and survival in the brain of rats would exist. These results indicated that MSCs retain their phenotype through several passages, and in this

way, increase the possibilities for clinical application of MSCs in treatment of different human diseases, which require substantial number of cells, a factor that makes the *ex vivo*-expansion of MSCs are very necessary (Hassan and El-Sheemy, 2004). Furthermore, MSCs transplantation in neurodegenerative diseases has been proven as feasible, safe and potentially effective. Although, there are doubts concerning the exact mechanisms, responsible for the beneficial outcome observed after MSCs transplantation into neurodegenerative tissue (Torrente and Polli, 2008; Li and Ikehara, 2013) have reported that MSCs are capable of secrete factors, including IL-6, IL-10, human growth factor (HGF), as well as platelet-derived growth factor (PGE2), that promote tissue repair, stimulate proliferation and differentiation of endogenous tissue progenitors, and decrease the inflammatory and immune reactions. From the current data, we can conclude that the intravenously-administered BM-MSCs enter brain and heart, and they survive, which might provide a cell source for treatment of stroke and heart diseases.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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