VERSITA

Biologia **69**/7: 863—869, 2014
Section Botany
DOI: 10.2478/s11756-014-0383-7

In vitro plant regeneration derived from leaf and stem explants of endemic *Thermopsis turcica*

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Abstract: This plant tissue study of micropropagation identifies the selective medium saving for rapid propagation in cultivated *Thermopsis turcica*, an endangered germplasm of the family Fabaceae. The aim is to obtain the optimum growth medium of *T. turcica* by enabling the *in vitro* propagation of this endemic. In this study, the leaves and stems of *T. turcica* were cultured on a Murashige and Skoog's medium supplemented with various concentrations (0.5, 1.0 and 2.0 mg L^{-1} 1-Naphthaleneacetic acid) of auxin and (0.2 and 0.5 mg L^{-1} Zeatin) (1.0 and 2.0 mg L^{-1} Benzylaminopurine) of cytokinins. Previous research focused on the regeneration from the seed of *T. turcica* Eber population; we concentrated upon the regeneration of different plant parts (leaf and stem) of *T. turcica* Aksehir population. In addition, according to the literature on *T. turcica* that to date the effects of Zeatin on the regeneration has not been performed. The most promising regeneration and growth were obtained from leaf explants cultured on the media with 2.0 mg L^{-1} 1-Naphthaleneacetic acid and 0.5 mg L^{-1}Zeatin (93.3%). The regenerated plantles were rooted on the media containing 2.0 mg L^{-1} Indole-3-butyric acid. Rooted plantlets were transplanted into potting of sterilized soil. The present study reports on the sufficient *in vitro* regeneration protocol through organogenesis in *T. turcica*. The findings presented here have implications for *in vitro* protection and use of this endemic endangered species in further biotechnological research.

Key words: *Thermopsis turcica*; micropropagation; growth medium; cytokinin; auxin

Introduction

The genus *Thermopsis*, which belongs to the Fabaceae (Leguminaceae) family, has 28 genera and 400 endemic plant species in Turkey (Erik & Tarikahya 2004; Vural 2009). *Thermopsis turcica* is only endemic representative of the genus *Thermopsis* in Turkey (Tan et al. 1983; Davis et al. 1988). The main agricultural trait of *T. turcica* is to have 2–3 free carpellate-ovaries, each containing 10 ovules. In addition, it has a long rhizome and 2–3 seeded legumes (Tan et al. 1983; Davis et al. 1988; Ozdemir et al. 2008). Furthermore, the species has been announced as critically endangered (CR) in the Red Data Book of Turkish Plants. However, due to seed predators that utilize *T. turcica* seeds for larval development as well as the loss of plant habitat owing to agricultural intensification, *T. turcica* is endangered (Ekim et al. 2000). Hence, there seems to be a need for *in vitro* preservation methods of threatened *T. turcica*.

In this study, sources of plant material were obtained from Istanbul's Nezahat Gokyigit Botanical Garden (NGBB). NGBB *T. turcica* conservation collection consists of two *T. turcica* populations, Eber and Aksehir, that have been obtained as populations from plants' natural habitat(s). However, ex-vitro conditions at NGBB may contain adverse environmental conditions including temperature, moisture and precipitation and consequently effect plant growth.

Because of these reasons, effective regeneration protocol is necessary to preserve this Turkish endemic endangered plant species *in vitro* conditions, as well. One of the major factors of *in vitro* plant propagation protocol is sufficient growth medium for selected plant species.

Research was conducted to adress the fact that little is known about the details of *T.turcica*'s developmental characteristics such as growth media and plant source, which might play an essential role in the microporopagation of the *T. turcica.*

The objective of the present study was to develop efficient and reliable *in vitro* regeneration protocol for endemic plant species of *T. turcica*. This regeneration protocol seeks to support conservation of this endemic species and further understand its biochemical characteristics.

Material and methods

Plant material

Different plant parts (leaf and stem) from Aksehir population of *T. turcica* were collected from Istanbul's Nezahat Gokyigit Botanical Garden, in late June of 2011.

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Table 1. Shoot regeneration, bulblets and callus formation percentages of explants on the medium containing different concentration of NAA (0.5, 1.0, 2.0, and 3.0 mg L^{-1}), Zeatin (0.2 and 0.5 mg L^{-1}) and BA (1.0 and 2.0 mg L^{-1}).

Explant	Plant Growth Regulators (mg L^{-1})			Percentage of shoots/explant	Percentage of bulblets/explant	Percentage of calli/explant
	NAA	BA	Zeatin			
Leaf tip	0	0	0	0	6.6	6.6
	0	1	0	6.6	0	26.6
	0	0	0.2	46.6	66.6	26.6
	0	2	0	0	0	0
	0	0	0.5	20	60	20
	0.5	0	0	0	6.6	6.6
	0.5	0	0.2	0	66.6	20
	0.5	0	0.5	0	13.3	46.6
	1	0	0	0	20	20
	1	1	0	0	33.3	80
	1	0	0.2	0	73.3	86.6
	1	2	0	26.6	33.3	100
	1	0	0.5	0	60	0
	2	0	0	0	6.6	13.3
	2	1	0	40	60	93.3
	2	0	0.2	0	53.3	60
	2	2	0	26.6	20	60
	2[1]	0[1]	0.5[1]	0	93.3	73.3
	3	0	0	0	20	20
	3	1	0	26.6	33.3	93.3
	3	2	0	26.6	6.6	60
Stem tip	0	0	0	0	0	6.6
	0	1	0	20	0	20
	0	0	0.2	26.6	20	60
	0	2	0	46.6	0	20
	0	0	0.5	26.6	13.3	73.3
	0.5	0	0	6.6	13.3	66.6
	0.5	0	0.2	26.6	20	73.3
	0.5[2]	0[2]	0.5[2]	26.6	53.3	86.6
	1	0	0	0	0	33.3
	1	1	0	66.6	20	60
	1	0	0.2	0	46.6	66.6
	1	2	0	13.3	6.6	80
	1	0	0.5	0	46.6	73.3
	2	0	0	0	6.6	86.6
	2	1	0	46.6	13.3	86.6
	2	0	0.2	0	46.6	86.6
	2	2	0	13.3	13.3	86.6
	2	0	0.5	0	40	60
	3	0	0	6.6	0	53.3
	3	1	0	0	40	60
	3	2	0	6.6	0	53.3

[1] The recommended regeneration medium content for leaf explants;
[2] The recommended regeneration medium content for stem explants

Media and culture conditions

In vitro grown explants were transferred into regeneration medium containing different concentration of 1-Naphthaleneacetic acid (NAA) (0.5, 1.0, 2.0 and 3.0 mg L^{-1}), Zeatin (0.2 and 0.5 mg L^{-1}) and Benzylaminopurine (BA) (1.0 and 2.0 mg L^{-1}) (Table 1) for organogenesis. The plants then were incubated at 25 ± 2°C under a 16 h photoperiod.

Surface sterilization

The explants (leaves and stems) were dipped into 70% ethanol and kept in 10% sodium hypochlorite for 5 minutes. The explants were then rinsed three consecutive times with sterile distilled water in a laminar flow cabinet (Dirks & Buggenum 1989; Kováč 1992).

In vitro culturing

After surface sterilization, the explants (leaf and stem parts) were placed into 100 × 15 mm petri dishes containing Murashige and Skoog's (MS) basal medium (Murashige & Skoog 1962) solidified by 7.0 g L^{-1} agar and added with different concentrations of NAA, BA, Zeatin, vitamins and 3% (w/v) sucrose. The pH of medium was adjusted to 5.8 before autoclaving. In addition, the regeneration medium contained MS salts, vitamins, and sucrose were used as the control. Cultures were incubated at 24 ± 1°C under 16-h photoperiod with 6000 lux by fluorescent. For each experiment, 30 explants were used. Each treatment was carried out in triplicate on groups of ten explants in each culture medium. The number of bud, callus and shoot per leaf and stem were determined weekly and explants were observed weekly by stereomicroscope for morphological observations. The regenerative ability of each explant (leaf and stem) was then scored weekly for a period of 6 weeks. Regeneration percentages for each explant were calculated (the (number of regenerated plants ÷ number of explants in culture) × 100). During microscopic observations, morpholog-

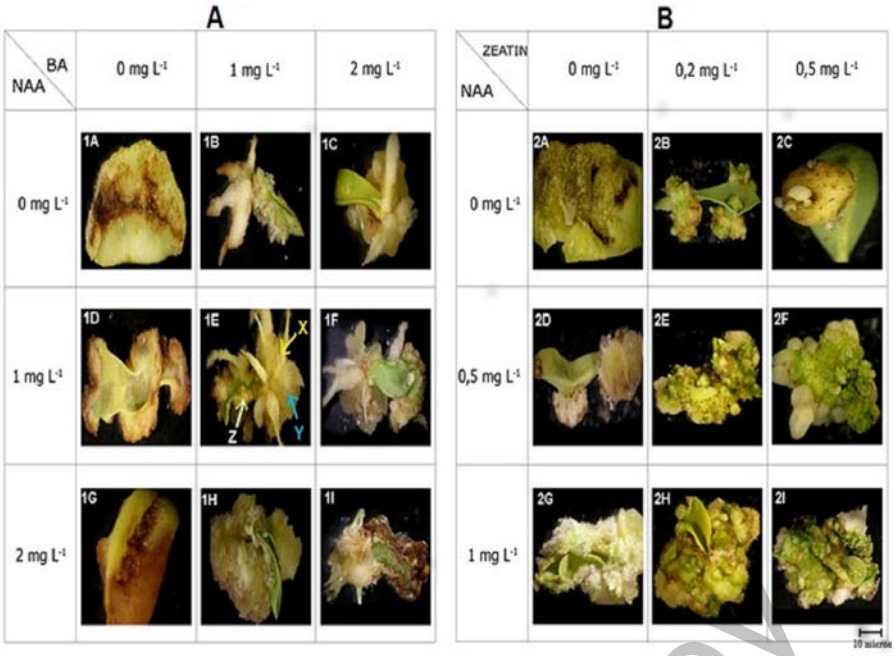

Fig. 1. Leaf explants at different stages of their development (arrows in 1E = X, root; Y, callus; Z, bud).

ical photographs of explants also were taken. Regenerating explants were subcultured with fresh medium.

Root development and acclimatization

The developing shoots (~1 cm) were excised and transferred to a rooting medium. As a rooting medium, MS basal medium containing 2.0 mg L^{-1} Indole-3-butyric acid (IBA) was used. All other cultural conditions remained as described above for *in vitro* culturing. After the rooted plantlets were acclimatized in a growth chamber, they were transplanted into pots containing autoclaved soil. Potted plants were grown under green house conditions for two months.

Results and discussion

Plant regeneration

Different concentrations and combinations of BA, NAA, and Zeatin were investigated to determine the optiumum regeneration medium for *T. turcica*. The evaluation of plant regeneration was carried out after 3 weeks. Regeneration data for *T. turcica* are presented in Table 1.

The number of shoot regeneration, bulblets, and callus formation varied according to the source explant. Control groups which developed on MS medium free from growth regulators did not show any growth (Figs 1-1A, 1-2A; 3-A1, 3-B1). On the other hand, explants cultured on the media containing growth regulators exhibited different growth responses according to the media tested. The best shoot regeneration percentage was found from stem explants cultured on the media containing 1.0 mg L^{-1} BA-1.0 mg L^{-1} NAA (66.6%), whereas leaf explants which developed on MS supplemented with 0.2 mg L^{-1} Zeatin alone produced the highest shoot regeneration response: 46.6 %. When the concentrations of NAA or BA were increased in the media, the frequency of shoot formation decreased. Percentages of bulblets regeneration reached 93.3% for leaf explants cultured on the media containing 2.0 mg L^{-1} NAA-0.5 mg L^{-1} Zeatin, but the percentage of bulblets response of stem explants cultivated on MS with 0.5 mg L^{-1} NAA-0.5 mg L^{-1} Zeatin was found at 53.3%.

Fig. 2. First leaves from leaf explants on MS medium supplemented with 1.0 mg L^{-1} NAA-0.2 mg L^{-1} Zeatin after 2 weeks in induction.

The best callus formation percentage was found from the media containing 2.0 mg L^{-1} BA-1.0 mg L^{-1} NAA: 100% for leaf explants, whereas the percentage of callus formation was 86.6% for stem explants cultured on MS media added (1) 1.0 mg L^{-1} BA-2.0 mg L^{-1} NAA, (2) 2.0 mg L^{-1} NAA alone, (3) 2.0 mg L^{-1} BA-2.0 mg L^{-1} NAA, (4) 2.0 mg L^{-1} NAA-0.2 mg L^{-1} Zeatin, and (5) 0.5 mg L^{-1} NAA-0.5mg L^{-1} Zeatin.

On media without growth regulators, control groups after 3 weeks of culture became brown and

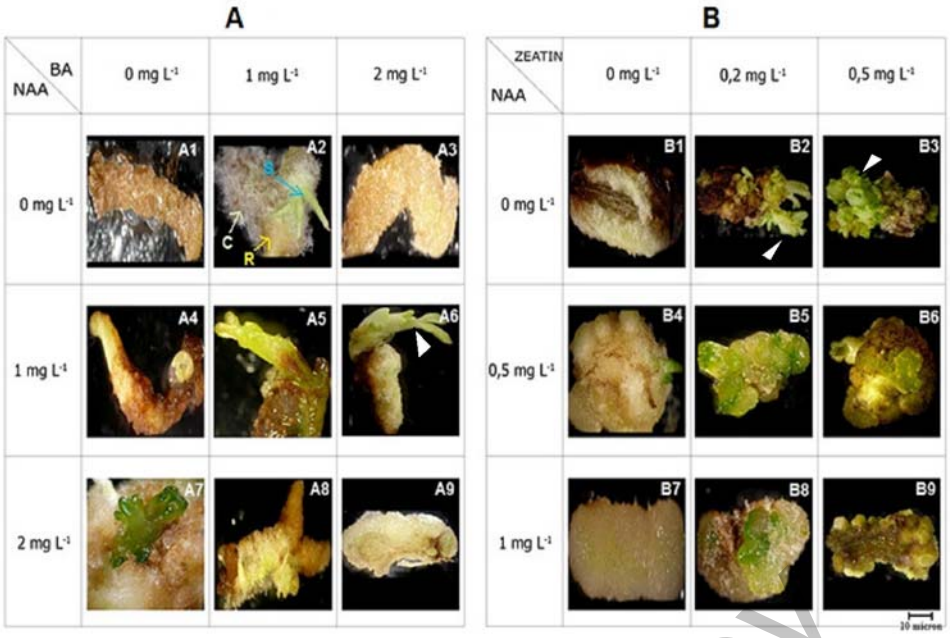

Fig. 3. Stem explants at different stages of development (arrows in A2 = R, root; C, callus; S, shoot and arrowheads: micro-shoots).

Fig. 4. First leaves from stem explants on MS medium supplemented with 1.0 mg L^{-1} NAA-2.0 mg L^{-1} BA (A); 0.0 mg L^{-1} NAA-0.5 mg L^{-1} Zeatin (B); 0.0 mg L^{-1} NAA-0.2 mg L^{-1} Zeatin (C) after 2 weeks in induction.

necrosed (Fig. 1-1A and 1-2A). On the other hand, shoot proliferating from *T. turcica* callus tissue *in vitro* was observed when the concentration of BA and NAA increased, but Zeatin treatments resulted in bulblet formation (Fig. 1). To date the effect of zeatin has been reported by different research groups (Dirks & Buggenum 1989; Kováč 1992; Malik & Saxena 1992; Gaba et al. 1999; Nasircilar et al. 2011); similar results were also described in these studies. The shoot regeneration of *T. turcica* on the MS medium containing different concentrations of NAA was also reported by Cenkci et al. (2008). The differences between the present study and that of Cenkci et al. (2008; 2009) were as follows: the concentrations and combinations of growth regulators used in the media, the source of the explant used for the research, and the selected population of *T. turcica*. Whereas Cenkci et al. (2008; 2009) used seed of the Eber population and rhizome of Aksehir population, we directly employed stem and leaf explants from the Aksehir population. According to the review of the literature on *T. turcica* to date, no study exists relating the effects of zeatin on the regeneration of this endemic rare species.

In the study presented here, the buds developed from the adaxial surface of leaf of *T. turcica*. By day 15, micro-shoots were observed. Finger-like protuberances were first observed in the MS medium with 1.0 mg L^{-1} NAA-0.2 mg L^{-1} Zeatin and leaves developed from these protuberances (Fig. 1-2H and 2). The occurence of callus was affected by explant and growth regulator type and also concentration. Formed calli showed differences in terms of their coloration and appearance (Fig. 1). Most were vigorous and white to yellowish in color.

Stem explants which developed on MS medium free

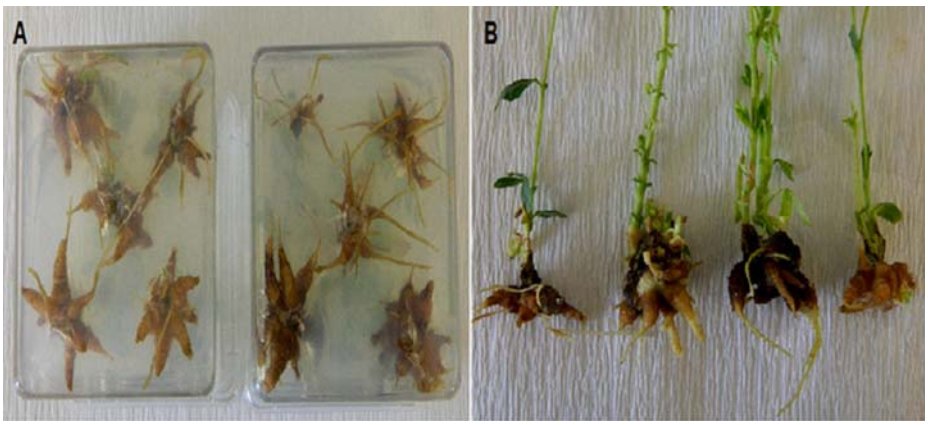

Fig. 5. *In vitro* root development on MS medium with 2.0 mg L^{-1} IBA. A – Rooted shoots in differing formation in magenta boxes (Magenta Corp., Chicago); B – rooting in regenerated *T. turcica* shoots.

Fig. 6. Rooted plantlets on MS medium with 2.0 mg L^{-1} IBA. A –Plantlets rooted in tubes; B – Plantlets rooted in magenta box.

from growth regulators did not grow and no buds were visible (Fig. 3).

On the media containing growth regulators, stem explants after 2 weeks of culture were observed. The greatest number of nodes on the first shoot was created on the medium with 1.0 mg L^{-1} BA-1.0 mg L^{-1} NAA (66.6%) (Fig. 3-A5). Callus formation from stem explants occured in response to all media tested (Fig. 3). Shoot meristems from stem explants gradually developed into leaves (Fig. 4).

Rooting and acclimatization

The regenerated plantlets were transferred to a MS medium containing 2.0 mg L^{-1} IBA for rooting, with the emergence of roots after 2 weeks. Cenkci et al. (2008) analyzed the effect of NAA and IBA on root development and reported that the best result for root formation was obtained from a MS medium supplemented with 0.5 mg L^{-1} IBA. In our study, 2.0 mg L^{-1} IBA was quite useful in obtaining differently characterized roots in terms of various aspect (short, long, thin, thicks etc.), and coloration range (dark and light colour) (Figs 5, 6). A good deal of research has stated that IBA is a robust source for rooting shoots as mentioned earlier in the literature (Adelberg et al. 1994; Mikulik 1999; Erdag & Emek 2009; Akin & Kocacaliskan 2011). The media used in the study presented containing IBA triggered root creation. This result as those of the previous studies show the effect of IBA on root formation.

In addition, we observed that the application of IBA induced some flower-like extensions (Fig. 7). Although IBA was found to be useful for flower induction in *T. turcica*, there was no particular information on the *in vitro* development of female and male gametophytes.

According to previous studies, auxins are essential for flowering (Rout & Das 1994; Franklin et al. 2000; Erdag & Emek 2009).

A few rooted plants continued to grow when trans-

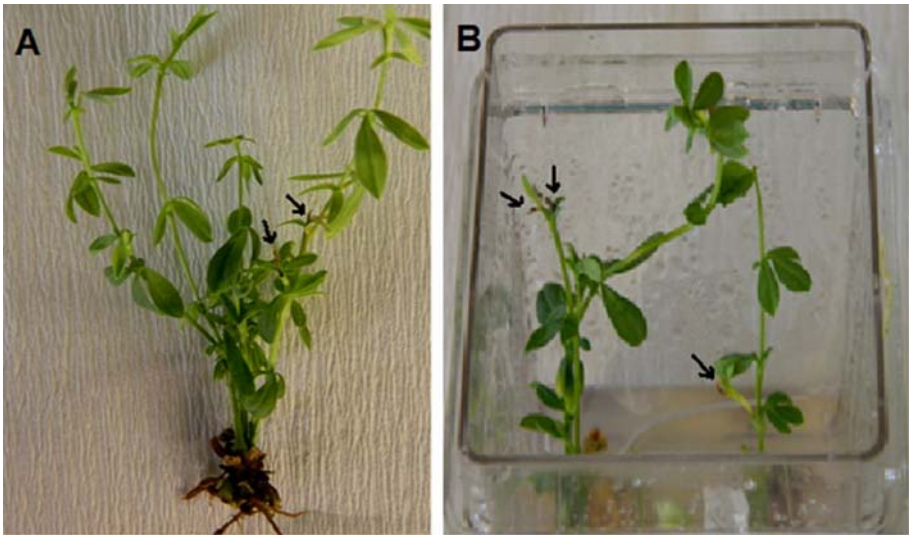

Fig. 7. *In vitro* flowering on magenta boxes including MS medium with 2.0 mg L^{-1} IBA (arrows = flower formations).

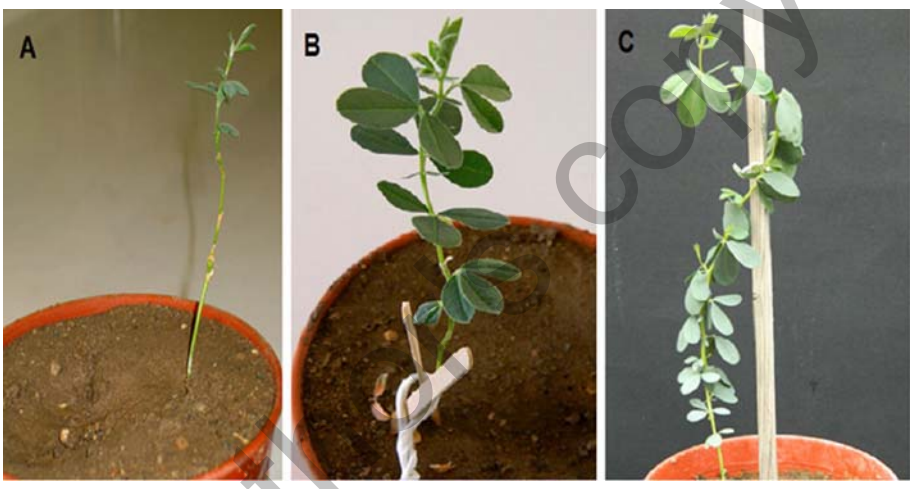

Fig. 8. Acclimatized plantlet in the greenhouse. A – Two-weeks old plant; B – A month old plant; C – Two-months old plant.

ferred in a greenhouse (Fig. 8) and after transferring to soil, the number of surviving plants did not exceed 10% for those issued from both two explants: leaf and stem. Cenkci et al. (2007) analyzed seedling developments and states that soil, perlit, and sand mixture (1:1:1) is suitable for growing of *T. turcica* in greenhouse conditions; in contrast, in that study, sterilized soil was used and few healthy plants obtained.

The transfer of plant to greenhouse occured in September and temperature condition could have affected the maintanance of plants under greenhouse conditions, as already claimed in Mikulik (1999).

We can conclude that we identified the selective medium saving for rapid propagation in cultivated *Thermopsis turcica*. *In vitro* plant regeneration was achieved from leaf and stem explants of *T. turcica*. Although rooted plantlets were tested to grow in *in-vivo* conditions, different growing parameters such as variations in soil samples were not conducted in detail. In addition, flower production of *T. turcica* under *in vitro* conditions was not tested in the present study. For these reasons, further studies are needed to analyze *in vitro* flower induction and seed production.

Acknowledgements

The authors are grateful to the Nezahat Gokyigit Botanic Garden, of Istanbul, for providing the germplasm used in the study. We also thank Nancy Karabeyoglu for critically reading this manuscript.

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Received October 31, 2013
Accepted March 28, 2014