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CONTENTS

AURELIA BREZEANU PhD.
75 YEARS OF LIFE AND 50 YEARS OF SCIENTIFIC ACTIVITY

CONSTANTIN TOMA[1]

A highly valuable intellectual personality, an outstanding scientist in Plant Biology domain, a founder of The Laboratory of Cell and Tissue "in vitro" Cultures – Institute of Biology, Romanian Academy, a trainer and school leader, Mrs. Aurelia Brezeanu PhD is one of the most valuable Romanian biologists in our days, well-known and recognized by Romanian and foreign specialists for her impressive scientific work performed with a huge passion and competence for the last 50 years. Her scientific activity was materialized in many books (12) and original scientific articles (575) published in famous scientific journals and publishing houses in our country and abroad, participations in many national and international professional meetings.

Aurelia Brezeanu PhD was born on the 11th of August, 1940 in Bucharest, in a modest intellectual family. She graduated from "Ion Creangă" High School and The Bucharest University – Faculty of Biolgy in 1962. She became a scientific researcher at the Institute of Biology of The Romanian Academy. She dedicated the last 50 years of her scientific activity to various areas of plant biology: morphology, cyto-histo-chemistry cyto-differentiation and morphogenesis, biotechnology as well as to genetic engineering and plant ecology.

In 1975, at the end of one year 1REX scholarship in the USA (Life Institute– Fargo N. Dakota and Beltswille University, Maryland) she started study "in vitro" the plants, cells and tissues cultures, and biotechnology. Since 1975, she has coordinated the team of researchers within a Plant Morphogenesis and Genetic Engineering Laboratory, newly created in the Institute of Biology in Bucharest on Acad. Gh. Zarnea's initiative.

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In 1981 she attended an international course on Plant Breeding and Propagation, organized by The British Council at the "John Innes Institute" in Norwich (England).

During over 40 years of activity in this field, several basic research directions have been developed that contributed to a better understanding of the biology of plant development like cyto-differentiation and morphogenesis, protoplast technology, a pioneer activity in Romania, gene transfer in plant cells mediated by bacterial plasmid vectors using direct (electroporation and electrotransfection) electrostimulation of plant growth and differentiation "in vitro" system by using weak electric fields.

A special attention was paid to studies of apoptosis and senescence processes in plant using "in vitro" culture to cellular proliferation and biosynthesis of secondary metabolites of biotechnological interest like anthocyanins, pycnogenol and resveratrol from some *Vitis vinifera* callus cell lines.

In the latest years she has consecrated her activity to biodiversity, "ex situ" conservation of rare, vulnerable and endangered plants from different taxa (bryophyta, lichens, ferns and vascular plants).

Aurelia Brezeanu PhD published as single author or in collaboration over 12 books, a few book chapters and about 575 original scientific papers both in Romania and abroad.

Some of them received prizes: "Emil Racoviţă" Prize (Romanian Academy, 1982) for the book "Ultrastructure of the Plant Cell – Atlas" (Anghel I., Brezeanu A., Toma N., 1981), The Prize of Academy of The Moldova Republic, 2004 for the book "Carpoculture in vitro" Nonmorphogenic pathway (Matienco B. *et al.*, 2004), the highest prize Fritzphil Prize, a scientific documentary film, produced by "Sahia" Film Studio in 1985 (Director Mircea Popescu and Scientific consultant Aurelia Brezeanu).

Her studies on plant cell biology were also appreciated by the international scientific comunity being included in "*Who's Who*" *The World Encyclopedia*, Ed. IV, 2009.

Under the scientific supervision of Aurelia Brezeanu PhD, over 42 doctoral theses in the field of cell biology and plant biotechnology were elaborated in the Institute of Biology.

I have met Mrs. Aurelia Brezeanu personally 30 years ago, as a member in The Board Council of the Central Institute of Biology, in The Board of periodical "Revue Roumaine de Biologie" (série de Biologie végétale), and, after a while, I have met her again as a member of The Board of "Romanian Journal of Biology – Plant Biology", or in some boards for promotion of young researchers. My colleague Aurelia Brezeanu has always been a person who gave a real and very important support to this journal through her demanding analysis of all scientific materials received for publishing.

Aurelia Brezeanu is a friend of biologists from Iaşi, a very close collaborator in our efforts to elaborate some anniversary or commemoration papers in the honour of some Romanian personalities in the Biology domain.

Mrs. Aurelia Brezeanu PhD has polarized around her the best graduates of biology faculties from Bucureşti, Cluj and Iaşi. She managed to create and deliver a real academic atmosphere, she has the certitude that a pupil cannot become a real good researcher if he does not bear the "sacred fire of scientific knowledge" inside of his soul.

Rightfully, Mrs. Aurelia Brezeanu PhD could be considered a model for those people who try to find a real model to follow during their lives. Students and co-workers appreciate her long experience and all those qualities hard to be identified to other schoolmasters: a huge capacity of work, passion, exigence, patience, generosity, solicitude, constructive ideas, team spirit, hopefulness, perspicacity, and a huge and open soul.

Distinguished colleague, at this anniversary moment I wish you from all my heart "Many and very good years" from this time forward alongside your family and collaborators!

MICROGREENS - A NEW FOOD PRODUCT WITH GREAT EXPECTATIONS

T. STOLERU[1], ALEXANDRINA IONIȚĂ[2], MARIA-MAGDALENA ZAMFIRACHE[1]

In an effort to alleviate some of the problems associated with the rising of human population and to make a step forward in ensuring food security, the vegetable industry has introduced microgreens – a new food product with good nutritional value and fast production rates. In this article we have explained what microgreens are, giving examples of the most used plant species to grow as microgreens and reviewed some of the scientific literature to provide an idea of the research directions undergone regarding microgreens. We wrote about growing microgreens, including substrate aspects, seeds aspects and grow light conditions. As well, we talked about nutritional quality of this food product and identified some disadvantages associated with microgreens production. In conclusion, even though individual and commercial productions are set to develop fast, health claims and nutritional facts should be thoroughly documented.

Keywords: Microgreens, food security, nutritional quality, microgreens disadvantages.

INTRODUCTION

The human population is steadyli rising, reachind numbers unimaginable o' a few centuries ago. The exponential growth of the past 200 years has resulted in a global number of over 7 billion people, exerting enormous pressure on the capabilities of modern agriculture (McClung, 2014). Although the percentage of people with insufficient access to food decreased considerably in the last 50 years, from 60% in 1960 to around 15% in 2010 (Godfray *et al*., 2010), there are currently about 1 billion people chronically malnourished and another approximately 2 billion people who suffer from a deficiency of essential micronutrients (Godfray *et al*., 2010; Godfray & Garnett, 2014). In this context and considering the projections and scenarios of an increase in population figures over 9 billion people by 2050 (Lutz & K C, 2010), it becomes imperative to focus our attention and efforts towards finding innovative means that can help alleviate the problem and ensure food security.

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To address the need of a diet with fresh, nutrient-rich and high content of phytocompounds necessary for healthy development of the body, the vegetables industry has introduced a new product: microgreens. The microgreens can be considered an innovation of the concept of vegetables and vegetables industry in general, having the potential to transform the whole idea of vegetables (Di Gioia & Santamaria, 2015).

Figure 1. Basil (*Ocimum basilicum* var. Limonero) microgreens 10 days after sowing.

Microgreens (Fig. 1), also known as "vegetable confetti" (Treadwell *et al*., 2010) or "microherbs" when referring to aromatic herbs (Di Gioia & Santamaria, 2015) are food products of vegetable origin. Although they are often described as culinary specialties used as an ingredient in kitchens of high-end restaurants, they are enjoying a growing popularity and an ever wider use as human food.

The microgreens are normal plants sown at a medium to high density and harvested shortly after the first true leaves emerge. Harvesting is done by cutting the stem just above the soil in which they grow or above the roots if the cultivation method is without soil. The time period from sowing to harvest is generally between 7 and 14 days, exceptionally reaching up to 21 days for some species with slower development, like celery. The harvested product is represented by this micro-plants with stems, cotyledonary leaves and the first (1-2) true leaves. Sometimes, depending on the species, the integument of the seed remains attached to the cotyledonary leaves and can be considered edible too (Di Gioia &

Santamaria, 2015). Microgreens are classified as a new category of vegetables, having different traits from the more commonly known sprouts and the early-cut leafy vegetables also known as baby greens.

The idea of microgreens can be traced back at the beginning of the 1980's when they were used in the menus of the chefs from the San Francisco area in California (Bliss, 2014). Commercial cultivation starts to take place in the second half of the 1990's in the Southern part of California (Di Gioia & Santamaria, 2015).

Microgreens possess a few advantages over the other categories of edible vegetables. Over sprouts which are usually produced in the dark with a very short production cycle, microgreens benefit of the surrounding environment, taking full use of light conditions and nutrients from the soil or alternative growing substrates. Over the baby leaf vegetables, which need to be harvested through a cut and then commercialized losing nutrients before reaching the final consumer, microgreens can be sold keeping the plantlets alive in the soil or the growing media and the final consumer can cut the product minutes before usage. This innovation of selling the product while it is still growing guarantees a longer shelf life and assures a high quality in terms of both freshness and nutritional value (Di Gioia & Santamaria, 2015).

From the three categories of product (sprouts, microgreens, baby leaf), only "sprouts" have a legal definition and their production and commercialization must comply with strict regulation because of their relatively higher risk of microbial contamination (Treadwell *et al*., 2010).

Crops that germinate easily and grow quickly are suitable for growing as microgreens. Microgreens include many common vegetables and herbs. Different types of microgreens include: cabbage, radish, turnip, carrot, beet, chard, pea, broccoli, kale, bok choy, celery, sesame, amaranth, cress, lettuce, endive, arugula, mustard, sunflower, alfalfa, clover, sorrel, canola, chia, flax, fennel, dill, basil, cilantro, and chervil. Microgreens are planted with the same seeds that are used to grow their full-sized counterparts. The basic concept of growing microgreens is harvesting the plants while they are young. Microgreens provide a large variety of flavours, colours and textures.

Di Gioia and Santamaria (2015) suggest that the production of microgreens from local varieties of traditional vegetables and the wild species would be more beneficial due to their higher nutrient content compared to commercial improved varieties. In this way the biodiversity would be preserved and valorised. Among the wild edible plants which may be considered for the production of microgreens, the authors suggest: common amaranth (*Amaranthus retroflexus* L.), blood amaranth (*Amaranthus cruentus* L.), borage (*Borago officinalis* L.), pigweed (*Chenopodium album* L.), wild chicory (*Cichorium intybus* L.), wild fennel (*Foeniculum vulgare* Mill.), common purslane (*Portulaca oleracea* L.), wild radish (*Raphanus raphanistrum* L.), white mustard (Sinapis alba L.), common dandelion (*Taraxacum officinale* Weber).

GROWING MICROGREENS

Microgreens can be grown at a small scale, by individuals for home use, or at a large scale, in industrial production systems, for commercial marketing. Growing, harvesting and postharvest handling may have a considerable effect on the accumulation and degradation of phytonutrients in microgreens.

Regarding the cultivating conditions, microgreens are a versatile product. They may be grown:

- in greenhouse or indoor,
- with natural or artificial light sources,
- in soil or in soilless systems.

Substrate aspects

The traditional soil cultivation for microgreens is recommended for individual growers but at large scale, hydroponic growing systems work better. In these systems different soilless growing media are used. The main soil substitute substrates used for microgreens production are peat-based mixes and synthetic mats. Since these types of substrates are expensive and non-renewable, scientists tried to find alternative solutions. Di Gioia *et al.* (2016) showed that beside polyethylene terephthalate and peat, other alternative cheap substrates can be used (textile fibre and jute-kenaf-fibre). The study was conducted on "rapini" microgreens (*Brassica rapa* L., Broccoleto group). Another conclusion of the study is that the choice of the growing medium represents one of the most critical aspects with a considerable impact on the productivity, quality and safety of microgreens.

Seeds aspects

The production of microgreens requires high quantities of seeds which imply high costs. Seeds used for microgreens must have a high quality, they should have a germination capacity over 95% and a good germinating power. Also the seeds must have a good percentage of purity and should be free of pathogenic bacteria or molds.

Because during time there were documented foodborne illness outbreaks caused by the sprouts contaminated with *Escherichia coli* O157:H7 nowadays their production is regulated by a number of international standards. In this respect there are no regulations regarding the microbiological safety of microgreens. Therefore the scientists examined the potential for growth of these bacteria in different microgreens production systems, using seeds inoculated with *Escherichia coli* O157:H7. The results demonstrated that bacteria grew both on the microgreen plants and in the growth substrata. The survival and proliferation of bacterial cells was higher in hydroponic production system than in soil substitute system (Xiao *et al.*, 2015). Another comparative study performed previously, which compared the

growth of this pathogen during sprout and microgreens production, concluded that a significant proliferation of *E. coli* O157:H7 and O104:H4 occurred during both sprouting and microgreens growth. This means that microgreens production should be subjected to the food safety standards applied to sprouts (Xiao *et al.*, 2014).

Light aspects

The light environment plays an important role in the development and accumulation of phytochemicals in microgreens. Some microgreens (dill, cilandro, chervil, argula, amaranth) grow best under indirect sunlight but artificial light also promotes plant growth and can be optimized to obtain the best results. There are many types of artificial sources of light used in microgreen production, among them the Light Emitting Diode (LED) appears to be the best choice. LEDs are designed to produce visible light and they have been shown to be an effective radiation source for plant growth (Bula *et al*, 1991). LEDs require the least amount of energy to produce light and very little heat, and are therefore the most efficient and cost effective comparing with other artificial sources.

Some studies were focused on enhancing the accumulation of nutritionally important shoot tissue pigments in leafy vegetables controlling the source of light. When speaking about artificial sources of light, the lighting spectra and the irradiance level are important parameters.

A team of Lithuanian scientists (Samuolienė *et al., 2*013) published a study regarding the influence of LED irradiance level on growth, nutritional quality and antioxidant properties of *Brassica* microgreens. They used high light stress to activate the photoprotection mechanism in microgreens. As a consequence, the production of antioxidants (anthocyanins, α-tocopherol and ascorbate) enhanced.

The study concluded that moderate light levels were more effective at enhancing the nutritional value of microgreens while high light exposure could cause detrimental effects on produce quality. Depending on the species, the most suitable conditions for growth and nutritional quality of microgreens was 320–440 μmol m^{-2} s^{-1}. To establish the optimal irradiance level scientists took into account both agronomic and economic aspect.

The study conducted by Lester *et al.* (2010) on baby leaf spinach (*Spinacia oleracea* L.) using different γ-irradiation doses found that continuous exposure prevented loss of ascorbic acid and was beneficial in enhancing the amounts of carotenoids and tocopherols.

The effect of source of light on microgreens was studied also by Kopsell *et al*. (2016). They compared the effect of fluorescent/incandescent light and blue/red LED light on the pigment content of 30-day-old Chinese kale (*Brassica oleracea* var. *alboglabra*). They found that sole source LED lighting produces higher concentrations of carotenoid and chlorophyll pigments in kale.

MICROGREENS NUTRITIONAL QUALITY

A number of studies over the last two decades demonstrated that the old saying "We are what we eat" is quite true. Sometimes our diet doesn't provide the body with all the nutrients, minerals, vitamins and antioxidants we need to be healthy. Frequently we resort to pills to solve the problem quickly and in a simple way. But are these supplements effective? Functional food with a moderate concentration of bioactive compounds seems to be by far better assimilated by our body than concentrated supplements (Meltzer, 2010).

Clinical trials as well as epidemiological studies showed that a plant based dietary with high intake of fruits and vegetables is associated with reduced risk of cancer, cardiovascular diseases and other chronic diseases. It seems that some bioactive plant compounds generically called antioxidants are responsible for this beneficial effect. The evidence that bioactive compounds in vegetables have important health related effects provides a strong motivation for people to increase the intake of fruits and vegetables. An important strategy to increase the intake of health related bioactive substances is to increase the concentration in food plants. In this respect, microgreens are a welcomed product on the market. Thus, microgreens belong to the group known as „functional food". Different microgreens contain widely differing amounts of functional compounds like antioxidants, minerals, vitamins and phenolics (Blomhoff, 2010). Growing, harvesting, and storage conditions may have a considerable effect on nutrient content.

One of the first studies related to the nutritional content of microgreens was performed by Xiao *et al.* and their results were published in 2012. The researchers assessed the concentration of vitamins and carotenoids in 25 microgreens. The highest concentration of vitamin C, carotenoids, phylloquinone and tocopherols were found in red cabbage, cilantro, garnet amaranth and green daikon radish.

The study concluded that microgreen cotyledon leaves possess higher nutritional value than the mature leaves. Researchers also found about five times greater levels of vitamins in microgreens than in their mature plant counterparts (Xiao *et al.*, 2012).

The researchers from Food Quality Laboratory and Crop Systems and Global Change Laboratory, USDA-ARS, conducted a study that analysed the concentrations of macroelements (calcium, magnesium, phosphorus, sodium, potassium) and of microelements (copper, iron, manganese, and zinc) of 30 species of microgreens from 10 genera of the *Brassicaceae* family. Results demonstrated that *Brassicaceae* microgreens are good sources of macroelements (e.g., potassium and calcium) and microelements (e.g., iron and zinc). This study seems to be the first to document mineral content of commercially available *Brassicaceae* microgreens (Xiao *et al*., 2016).

The protective effect against the oxidative stress shown by *Brassicaceae* (broccoli, Brussels sprouts, cabbage, kale, cauliflower) is given by glucosinolates

which are sulphur-containing glucosides. For example in broccoli there are: sinigrin, glucoraphanin and progoitrin; in Chinese cabbage there is indolyl glucosinolate glucobrassicin, and glucoraphanin is one of the most abundant glucosinolates present in broccoli. Vegetables from *Brassicaceae* family are known to contain also high concentrations of polyphenols associated with human health: anthocyanins, flavonol glycosides, hydroxycinnamic acids, etc. Assuming that microgreens are more nutritious than the mature plants, Sun *et al.* (2013) conducted a comparative study in five *Brassica* species. The results showed that microgreens contain more variety of complex polyphenols than mature plants. There were identified 164 polyphenols from which 30 were anthocyanins, 105 were flavonol glicosides and 29 were hydroxycinnamic and hydroxybenzoic acid derivatives thus proving that microgreens are an important source of bioactive substances.

Lettuce contains various health-promoting phytochemicals, including vitamins and phenolic compounds with antioxidant properties. Oh *et al.* (2010) found that young lettuce (*Lactuca sativa*) seedlings after 7 days of germination had the highest total phenolic concentration and antioxidant capacity in comparison to the mature leaves.

For most of the people, when speaking about food, equally or maybe more important than the nutritional value are the sensory attributes. Appearance, texture and flavour are the main sensory attributes to evaluate the quality of fresh products. The chemical composition and sensory qualities (sweetness, bitterness, astringency, sourness, heat) of some microgreens were evaluated in a study conducted by Xiao *et al.* (2015). Six microgreen species were evaluated: Dijon mustard (*Brassica juncea* L. Czern.), opal basil (*Ocimum basilicum* L.), bull's blood beet (*Beta vulgaris* L.), red amaranth (*Amaranthus tricolor* L.), peppercress (*Lepidium bonariense* L.) and China rose radish (*Raphanus sativus* L.). The researchers found a strong correlation between the chemical composition (total phenolic content) of the microgreens and their flavour attributes. The chemical analysis revealed that China rose radish, opal basil and red amaranth have the highest concentrations of total ascorbic acid, phylloquinone, carotenoids and tocopherols while the highest concentrations of total phenolics were found in China rose radish, and opal basil. The study also concluded that pH and total phenolics values could be used by microgreen growers as indicators and predictors of consumer acceptability. Unfortunately the results of the study reflected the reality that people reject some vegetables because of their unpleasant flavour (bitterness, astringency) even if they have beneficial effect on human health.

DISADVANTAGES ASSOCIATED WITH MICROGREENS PRODUCTION

- Short shelf life with the reduction in nutritional value during storage
- The risk of contamination with pathogens and public health

Industrial production and marketing of microgreens is limited by their short shelf life associated with rapid deterioration in product quality. There are various

pre and postharvest techniques applied currently to extend the shelf life of microgreens. The techniques applied after harvesting are related to the storage condition and imply a strict control of temperature and atmospheric composition. So, the shelf life of microgreens depends on many factors such as temperature, relative humidity, packaging film type and microbial load. Researchers tried to find solutions to avoid the deterioration of quality during postharvest storage.

The scientists from the Food Quality Laboratory of the USDA-ARS determined that daikon radish microgreens keep the nutritional value when using film bags highly porous to oxygen. Another condition to prolong the shelf life is storage in darkness at refrigeration temperature during distribution and retail.

Related studies were performed by Kou *et al.* (2013) to extend the shelf life of buckwheat microgreens. Although buckwheat microgreens are rich in antioxidants and vitamins their short shelf life has limited their commercial use. This study tried to optimize the storage conditions so that to preserve the nutritional quality of the product. It was found that a temperature between 5 and 10 °C and an atmosphere with moderately high O_2 (14.0-16.5 kPa) and moderately low CO_2 (1.0-1.5 kPa) levels is optimal for a maximal shelf life. The researchers also fine-tuned packages to provide the optimal atmospheric composition required to extend the shelf life of buckwheat microgreens.

But the shelf life of microgreens can also be extended using some pre-harvest techniques. Kou *et al.* (2014) investigated the effect of pre-harvest calcium application on the quality of broccoli microgreens. The post-harvest quality and the shelf life of the treated microgreens increased to 21 days. The non-treated microgreens were edible only 14 days. A dose of 10 mM of calcium chloride increased biomass production (more than 50%), enhanced the activity of superoxide dismutase and peroxidase in microgreens and significantly reduced the microbial growth during storage. The treatment also tripled the concentration of calcium in microgreens.

In practice, to prevent the microbial growth in the post-harvest stage, the microgreens are washed with chlorinated water, in doses that do not affect the taste and flavour of the product (Mir *et al*., 2016). Chandra *et al*. (2012) studied the effects of different sanitizing agents on the quality and microbial population of *Brassica campestris* var. farinosa microgreens. The scientists used in different combinations the following sanitizers: chlorine, citric acid, ascorbic acid and ethanol spray. The results suggest that the combination of citric acid with ethanol spray could replace successfully the chlorine commonly used for washing of microgreens.

CONCLUSIONS

A healthy and well-balanced diet seems to be the basic strategy to obtain a good health. Without a balanced diet as a basis, functional foods like microgreens will probably have less impact on public health.

There's still a lot of debate regarding the health benefits derived from microgreens consumption. Some consider that there are not sufficient scientific data to prove the higher level of nutrients in microgreens than in mature plants. But as revealed from this review, for sure the production of microgreens is a great addition in horticulture. Growing microgreens can be very productive and this advantage can be used to solve the growing demand of food. However, microgreens industry will increase but health claims and nutritional facts of these products should be thoroughly documented.

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CHOROLOGY OF *PARMELIA* GENUS IN ROMANIA

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The paper presents data about the distribution of *Parmelia* genus in Romania. The work is based on original, herbarium and literature data. It was observed that two species from this genus are widespread in Romania, namely: *Parmelia saxatilis* and *Parmelia sulcata*. Also, worldwide data support the statement mentioned above. As regards the other species tabulated within *Parmelia* genus, these have a limited distribution on the Romanian territory.

Keywords: chorology, *Parmelia*, Romania.

INTRODUCTION

Parmelia genus was designed by Acharius in 1803 (Hale, 1987) and is represented by more than 71 species (Sharma *et al.* 2013) widespread in the both boreal and austral hemispheres (Hale, 1987; Thell *et al*., 2012). The studied genus is paraphyletic and its genetic centre is in the northern hemisphere (Thell *et al.* 2012).

Data about the general distribution of the *Parmelia* genus are highlighted within a lot of papers. Thus, the mentioned genus was cited from the following continents:

Africa: **Ethiopia**, **Kenya** (Hale, 1987), **Morocco** (Molina *et al*. 2011), **South Africa** (Brusse, 1984; Hale, 1987), **Tunisia** (Hale, 1987).

Antarctica: *Palmer Peninsula, Graham Coast, Tuxen Cape* (Hale, 1987).

Asia: **Azerbaijan** (Alverdiyeva, 2014), **China** (Hale, 1987), **India** (Hale, 1987; Sharma *et al*. 2013), **Japan** (Hale, 1987; Molina *et al*. 2011), **Korea**, **Mongolia**, **Nepal** (Hale, 1987), **Russia** (Hale, 1987; Thell *et al*., 2008; Molina *et al.*, 2011; Sonina, 2012), **Malaysia**: *Sabah* (Hale, 1987), **Turkey** (Hale, 1987; Türk *et* Güner, 1998; Oran et Öztürk, 2012).

Australia: Shirley (1918); Hale (1987); Cranfield (2004); Morley et Gibson (2010).

Europe: **Austria** (Hale, 1987; Thell et al. 2008), **Belgium** (Hale, 1987; Thell *et al.* 2008), **Bosnia-Herzegovina** (Thell *et al.* 2008), **Bulgaria**, **Czech** (Hale, 1987), **Denmark**, **Estonia** (Degelius, 1986; Hale, 1987; Suija *et al.* 2008; Thell *et al.* 2002; Thell *et al.* 2008; Thell *et al*. 2012), **Finland**, **France** (Hale, 1987; Thell *et al*. 2002;

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Thell *et al*. 2008), **Georgia** (Hale, 1987; Inashvili et Batsatsashvili, 2010), **Germany** (Hale, 1987; Molina et al. 2011; Thell *et al.* 2008; Wirth, 2008), **Greece** (Hale, 1987), **Greenland** (Wetmore, 1968; Hale, 1987), **Hungary** (Hale, 1987), **Italy** (Hale, 1987; Thell *et al.* 2008), **Letonia** (Mežaka *et al.* 2008), **Lithuania** (Suija *et al*. 2008), **Luxembourg** (Thell *et al.* 2008), **The Netherlands** (Hale, 1987; Thell *et al.* 2008), **Norway** (Degelius, 1982; Hale, 1987; Suija *et al.* 2008), **Poland** (Hale, 1987; Ossowska *et al.* 2014), **Portugal** (Hale, 1987; Molina et al. 2011), **Scandinavian Peninsula** (Moberg and Holmåsen, 1992), **Slovakia**, **Spain** (Hale, 1987; Barreno and Herrera-Campos, 2006-2009; Thell *et al*. 2008; Molina *et al*. 2011), **Sweden** (Hale, 1987; Suija *et al*. 2008; Thell *et al*. 2002; Thell *et al.* 2008), **Switzerland** (Hale, 1987), **Ukraine** (Thell *et al.* 2008) **United Kingdom**: *England, Scotland* (Hale, 1987; Suija *et al*. 2008; Thell *et al.* 2008; Molina *et al*. 2011).

America: **Argentina** (Hale, 1987; Calvelo and Adler, 1999), **Chile** (Hale, 1987; Thell *et al*. 2002; Thell *et al*. 2008); **Alabama**, **Alaska, Arizona**, **Arkansas** (Wetmore, 1968; Hale, 1987), **California** (Wetmore, 1968; Hale, 1987; Molina *et al.* 2011), **Canada, Carolina**, **Colorado**, **Connecticut**, **Dakota**, **Florida**, **Idaho**, **Illinois**, **Iowa**, **Kentucky**, **Louisiana**, **Maryland** (Wetmore, 1968; Hale, 1987), **Maine**, **Massachusetts** (Wetmore, 1968; Hale, 1987; Molina *et al*. 2011), **Michigan, Minnesota** (Wetmore, 1968; Hale, 1987), **Montana** (Hale, 1987, Molina *et al*. 2011), **New Jersey** (Hale, 1987), **New Hampshire** (Wetmore, 1968; Hale, 1987; Molina *et al.* 2011), **New Mexoco**, **New York**, **Oregon** (Wetmore, 1968; Hale, 1987), **Ohio** (Conan, 1967; Flenniken and Showman, 1990), **Oklahoma** (Wetmore, 1968), **Pennsylvania** (Hale, 1987), **Tennessee** (Wetmore, 1968; Hale, 1987; Molina *et al.* 2011), **Vermont** (Wetmore, 1968), **Virginia**, **Washington**, **Wisconsin**, **Wyoming** (Wetmore, 1968; Hale, 1987), Southern Rockies Alleghenian and Great Lakes, Smoky Mountains, Black Hills (Wetmore, 1968).

Islands: **Adelaide**, **Campbell**, **Canary**: *Teneriffe*, *La Palma*, **Diomede**, **Falkland**, **Harbour**, **Indonesia**, **Kerguelen**, **Kurile**, **Macquarie**, **Marion**, **Navarino**, **New Zealand**, **Pandora**, **Philippines**, **Rhode**, **Saghalien** (Wetmore, 1968; Hale, 1987), **Taiwan** (Hale, 1987; Lai, 2001), **Tasmania** (Hale, 1987; Kantvilas, 1989).

It is well known that *P. saxatilis* and *P. sulcata* are "the most widespread species present in cold-temperate areas of both hemispheres. Some of the other species have restricted or poorly known distributions" (Thell *et al*. 2008; Thell *et al*. 2012).

The relevance of the study is given by the original data and also by an important and valuable background regarding the spatial distribution on this genus in Romania (literature and herbarium data). The book written by Ciurchea (2004) represents a synthesis paper of a lot of old literature data regarding, inter alia, chorology of lichen species in Romania.

The aim of this study consists in knowledge of *Parmelia* genus chorology on the Romania territory. The objective of this study is based on mapping of considered genus in Romania and carried out an update database regarding *Parmelia* genus distribution in Romania.

MATERIAL AND METHODS

The field activity was conducted during 2009-2015 in different habitats (natural and seminatural forests, alpine meadows and bog peat) from Romania. The chorology of *Parmelia* genus in Romania was carried out using UTM codes (Lehrer and Lehrer, 1990).

This work is based on data found within literature review, herbarium specimens preserved within The Mycological Herbarium of the Institute of Biology from Bucharest and original field data. The field identified material is preserved in the Mycological Herbarium, BUCM L – BUC (Bucharest), M (Mycological), L (Lichen) of the Institute of Biology of the Romanian Academy, Bucharest.

A stereomicroscope (Zeiss Stereo CL 1500 ECO) was used to lichen species determination. The lichen species were identified using determination keys (Moruzi and Toma, 1971; Ciurchea, 2004). The collected lichen species were determined by using KOH, Cl, Pd and $CaCl_2$ (Moruzi and Toma, 1971). The nomenclature of lichen species is in compliance to www.mycobank.org. The host tree's nomenclature is according to Ciocârlan (2009).

RESULTS AND DISCUSSION

In Romania the distribution of the 6 species belonging to *Parmelia* genus was mapped. The results have indicated that *P. sulcata* and *P. saxatilis* are the most widespread. A weakly spread was observed in case of the following lichen species: *Parmelia borreri* (Sm.) Turner 1807, *Parmelia pokornyi* (Zahlbr.) Szatala 1932 and *Parmelia submontana* Hale 1987. According to field observation the cover of *P. borreri, P. saxatilis* and *P. sulcata* is rather lower; however these lichen species are well represented especially in protected areas from Romania. Within unprotected areas the above mentioned lichen species are really scarce. Published data pointed out that *P. saxatilis* and *P. sulcata* are the most widespread lichen species in Romania (Cretzoiu, 1941).

The concerns on *Parmelia* genus chorology in Romania are presented as follows:

1. Parmelia *discordans* Nyl. 1886

Vâlcea County: (KL 92) Cozia National Park (Çobanoğlu *et al*. 2011).

2. *Parmelia omphalodes* (L.) Ach. 1803

Bacău County: (MM 70) Mănăstirea Caşin, Pucna, Piatra Runcului, Scutaru and Baba Mărioţei (Ciurchea, 2004).

Bistriţa-Năsăud County: (LN 36) Rodnei Mountains, Ineu Peak Valley (Zschacke, 1911; Moruzi *et al.* 1967); Rodnei Mountains National Park, Pietrosul Mare, lat. 47.5955°N, long. 24.6505°E, alt. 2136 m, Repedea Valley, lat. 47.5952°N, long. 24.679°E, alt. 1326 m, Gropile, lat. 47.579°N, long. 24.6325°E, alt. 2085 m (Ardelean *et al*. 2013).

Caraş-Severin County: (FQ 17) Băile Herculane (Moruzi *et al*. 1967; Ciurchea, 2004).

Cluj County: (FS 56) Someşului Cald Valley between Fântânele Dam (Beliş locality) and Tarniţa (Ciurchea and Crişan, 1991-1992; Ciurchea, 2004).

Dâmboviţa County: (LL 70) Bucegi Mountains, Cocora, leg. 1927 [BUCM L 0830].

Hunedoara County: (FR 42) Retezat Mountains in Zănoaga (Moruzi and Mantu, 1962; Ciurchea, 2004), near Petroşani town (Moruzi *et al.* 1967); Retezat National Park at Şeaua Retezatului, Faţa Retezatului, Creasta Zănoagei, Culmea Şeselor, Gemenea Lake, Tăul Negru and Zlătuia Valley (Ciurchea, 2004); (FR 85) Şureanu (Sebeş) Mountains on Şureanu Peak (Moruzi *et al.* 1967; Ciurchea, 2004).

Maramures County: (LN 27) Maramureşului Mountains, Pietrosul Mountain and Borşa Mountain (Moruzi *et al.* 1967; Ciurchea, 2004).

Mureş County: (LN 50) Neagră Brook, Landscape Reserve Defileul Deda-Topliţa (Crişan and Banc, 2007).

Sibiu County: (LL 15) Făgăraş Mountains, Arpaş Mountain (Moruzi *et al.* 1967; Ciurchea, 2004).

3. *Parmelia pokornyi* (Zahlbr.) Szatala 1925

Tulcea County: (PL 91) Letea Forest (Moruzi *et al*. 1967; Ciurchea, 2004); (NL 60) Măcin Mountains, leg. Vicol Ioana, 22.05.2013, det. Lőkös László, 27.08.2013 [BUCM L2282].

4. *Parmelia saxatilis* (L.) Ach. 1803

Alba County: (FR 85) Şureanu (Sebeşului) Mountains, Dealul lui Brat (Ciurchea, 2004); (GR 08) Dumbrava la Laz, Sebeşului Valley (Borza, 1959; Ciurchea, 2004); (FS 92) Galda de Sus, Bradu; (FS 33/43) Avram Iancu (Ciurchea, 2004); (GS 03) Bichiş, Rărişti, Lopadea, Vălişoara forests, in the neighbourhoods of Aiud (Györkös and Bartók, 2006).

Bihor County: (FS 27) Codru Mountain, Arsura Mountain, alt. 930 m (Gyelnik, 1928; Ciurchea, 2004), Bihorului Mountains on the Răchita Peak (Bartók, 1982; Ciurchea, 2004); (ET 61/70/71) Pădurea Craiului Mountains (Crişan, 2003).

Bistriţa-Năsăud County: (LN 36) Rodnei Mountains, Ineu Peak Valley (Zschacke, 1911; Moruzi *et al.* 1967; Ciurchea, 2004), Rodnei Mountains National Park, Borşa 1, lat. 47.6224°N, long. 24.6396°E, alt. 1048 m, lat. 47.6287°, long. 24.6635°E, alt. 1988 m, Pietrosul Mare, lat. 47.5955°N, long. 24.6505°E, alt. 2136

m, lat. 47.5935°N, long. 24.6407°E, alt. 2171 m, lat. 47.6004°N, long. 24.6231°E, alt. 1916 m, lat. 47.6028°N, long. 24.6054°E, alt. 1477 m, Repedea Valley, lat. 47.5952°N, long. 24.679°E, alt. 1326 m, Gropile, lat. 47.5703°N, long. 24.6468°E, alt. 2020 m, lat. 47.5739°N, long. 24.6344°E, alt. 1085 m, Bătrâna, lat. 47.5699°N, long. 24.6124°E, alt, 1915 m, Bătrâna, lat. 47.568°N, long. 24.6116°E, alt. 1816 m (Ardelean *et al*. 2013); Rebra Valley (Ciurchea, 2004).

Botoșani County: (MP 41) Pădureni Forest (Burlacu, 1967; Ciurchea, 2004); (MP 50) Horlăceni Forest (Burlacu, 1969b; Ciurchea, 2004).

Brașov County: (LM 83) Băile Homorod (Ciurchea, 2004); (LL 96) Cetății-Lempeș Hill, leg. Vicol Ioana, 18.03.2011, det. Vicol Ioana, 07.04.2011, [BUCM L1630].

Bucharest: (MK 21/22/31/32) Băneasa Forest, leg. Vicol Ioana, 18.03.2011, det. Vicol Ioana, 08.04.2011, [BUCM L1631], Vicol (2011c).

Buzău County: (ML 43) Buzău Mountains on Carpenul Peak (Moruzi *et al*. 1967; Ciurchea, 2004).

Caraș-Severin County: (FQ 17) Cernei Valley, Băile Herculane, Domogled Mountain (Moruzi *et al*. 1967; Ciurchea, 2004), (EQ 84) Cozla, Pescari (Burlacu *et al*. 1969; Ciurchea, 2004); (EQ 77) Banatului Mountains, leg. Dumitrescu Florentina, 19.11.2015, det. Vicol Ioana, 10.12.2015, [BUCM L2818].

Cluj County: (FS 56) Fântânele Dam, Beliș village, Culmea Ghiduri, Lunca III (Ciurchea, 1977; Ciurchea, 2004); Someșului Cald Valley between Fântânele Dam, Beliș village and Tarnița (Ciurchea and Crișan 1991-1992; Ciurchea, 2004); (FS 38) Vlădeasa Mountain (Bartók and Codoreanu, 1979), Preluca Rabului, (Ciurchea, 2004); (FS 38/48) Răcad Valley (Bartók, 1988; Ciurchea, 2004); (FS 87/88) Gilău Mountains, (FS 75) Mare Mountain at Capul Dealului (Bartók, 1989a; Ciurchea, 2004); (FS 97/98/GS/08) Cluj-Napoca in Botanical Gardens (Codoreanu *et al*. 1960; Moruzi *et al*. 1967; Ciurchea, 2004).

Dâmbovița County: (LL 51) Leaota Mountains (Tâncova Mountain and Romanescu Mountain) (Ciurchea, 2004).

Giurgiu County: (LK 92) Căscioarelor Forest, leg. Vicol Ioana, 17.03.2011, det. Vicol Ioana, 08.04.2011, [BUCM L1632]; (MK 02) Crevedia Forest, leg. Vicol Ioana, 28.03.2011, det. Vicol Ioana, 08.04.2011 [BUCM L1636].

Gorj County: (GR 02) Parâng Mountains, between Rânca and Obârșia Lotrului, on saxicolous substrata, leg. Vicol Ioana, 23.10.2012, det. Vicol Ioana, 07.11.2012, [BUCM L1836].

Harghita County: (LM 83) Harghita Mountains, near Căpâlnița locality (Moruzi *et al*. 1967; Ciurchea, 2004), Băile Homorod, Vlăhița (Barth, 1905; Moruzi *et al*. 1967); (LN 80/90) near Borsec locality, lat. 46.96561°N, long. 25.58633°E, alt. 790 m, leg. Vicol Ioana, 17.09.2015, det. Vicol Ioana, 28.09.2015, [BUCM L2687].

Hunedoara County: (FR 42) Retezat Mountains at Râușor Valley (Szatala, 1932, Moruzi *et al*. 1967; Ciurchea, 2004), Stănișoarei Valley, Bucura Lake,

Netedul Mountain, Câmpușel, Scorota cu apă, Scocul Iarului, near Pietrele Chalet (Moruzi and Mantu, 1962; Bartók, 1987b; Bartók, 1987c; Bartók, 1989b; Bartók, 1993; Ciurchea, 2004); (FR 42) Retezat National Park (Bartók, 1987b; Bartók, 1987c; Ciurchea, 2004); (FR 83) Jiețului Valley near Petroșani town (Cretzoiu, 1940; Ciurchea, 2004); (FR 85) Șureanu Mountains on Șureanu Peak, Aușelul Mountain, Surianul Mountain, Brat Mountain (Moruzi *et al.* 1967; Ciurchea, 2004); (FR 55) Bison Reserve Silvuț-Hațeg, leg. Vicol Ioana, 12.04.2015, det. Vicol Ioana, 23.04.2015 [BUCM L2630].

Iași County: (NN 31) Cornești Forest (Moruzi *et al.* 1967; Ciurchea, 2004).

Ilfov County: (MK 13) Mogoșoaia Forest (Moruzi and Mantu, 1965; Moruzi *et al*. 1967; Ciurchea, 2004), leg. Negrean G., 14.02.1960 [BUCM L0031]; (MK 34/35) Snagov Forest (Mantu, 1965; Moruzi *et al*. 1967; Ciurchea, 2004); (MK 24) Vlădiceasca Forest, leg. Vicol Ioana, 26.04.2010, det. Vicol Ioana, 11.05.2010, [BUCM L1320], Vicol (2011b).

Mureș County: (LN 22) Călimani Mountains on the Voivodesii Peak (Moruzi *et al*. 1967; Ciurchea, 2004); (LN 50) Neagră Brook, Landscape Reserve Defileul Deda-Toplița (Crișan and Banc, 2007).

Neamț County: (MN 43) Neamț Monastery (Moruzi *et al.* 1967; Ciurchea, 2004), leg. 1929, [BUCM L0225]; (MM 39) Tarcău Mountains on Tărhăuș Peak (Rotărescu-Burlacu, 1963; Moruzi *et al.* 1967; Ciurchea, 2004); (MM 18) Cheile Bicazului (Burlacu, 1969a; Ciurchea, 2004); (MN 20) Ceahlău Mountain at Horștei Peak, Poiana Ciribuc, Bâtca Durăului, Polița cu crini (Manoliu et al. 1998).

Prahova County: (ML 13) Ciucaș Mountains (Ciurchea, 2004); (LL 73) Bucegi Mountains (Mantu, 1967; Moruzi *et al*. 1967), Furnica Mountain (Zamfir *et al*. 1998).

Sibiu County: (LL 15) Făgăraș Mountains, Netedul Peak, alt. 2350 m, Cârțișoara Mountain, Vârtopelul, Arpaș Mountain, alt. 2200 m (Gyelnik, 1928; Codoreanu, 1957; Ciurchea, 2004) Budislavul, Ciortea, Negoiul Peak, Bradul (Moruzi *et al*. 1967; Ciurchea, 2004) (LL 15) Bâlea Waterfall (Ciurchea and Codoreanu 1967); (LL 17) Arpașului Valley; (KL 76) Măgura Mountain near Gureni (Zschacke, 1913; Moruzi *et al.* 1967; Ciurchea, 2004); (LL 17) Tătarilor Lake Nature Reserve, leg. Vicol Ioana, 29.03.2011, det. Vicol Ioana, 05.04.2011, [BUCM L1633]; leg. Vicol Ioana, 29.03.2011, det. Vicol Ioana, 31.03.2011 [BUCM L1635]; (KL 85/86) Tălmaciu Forest, leg. Vicol Ioana, 28.03.2011, det. Vicol Ioana, 05.04.2011 [BUCM L1634].

Suceava County: (LN95) Muntele Rarău, Schitu Rarău; (MN 45) Dealul Muncel (Moruzi *et al*. 1967; Ciurchea, 2004); Culmea Cioflăului, Pietrele Muierilor, alt. 920 m, (Gușuleac, 1930; Moruzi *et al.* 1967; Ciurchea, 2004); (LN 95) Codru Secular Slătioara (Ștefureac, 1941; Moruzi *et al*. 1967; Ciurchea, 2004); (LN 95) Pietrosul Broștenilor Mountain (Moruzi *et al.* 1967; Ciurchea, 2004); (LN 84) Bistriței Mountains, Pietrosul Bistriței Peak (Popa, 2006; Mardari (Popa) 2008).

Tulcea County: (NL 90) Culmea Pricopanului (Moruzi and Mantu, 1966; Ciurchea, 2004); (PK 89) Caraorman Grind, Danube Delta (Toma and Covaliuc, 2006).

Vaslui County: Cazacul Forest (Moruzi *et al*. 1967; Ciurchea, 2004); (NM 71) Măluşteni Nature Reserve, leg. Vicol Ioana, 21.05.2009, det. Vicol Ioana, 22.07.2009 [BUCM L1417], Vicol (2011a)

Vâlcea County: (KL 82) Lotrului Mountains, Călineşti Valley (Ciurchea, 1970; Ciurchea, 2004); (LL 79) Cozia Defile, Oltului Valley, between Proeni and Călineşti (Ciurchea, 1969; Ciurchea, 2004); (KL 92) Cozia National Park (Çobanoğlu *et al*. 2009).

5. *Parmelia submontana* Nádv. ex Hale

Vâlcea County: (KL 92) Cozia National Park (Çobanoğlu *et al*. 2009).

Bistriţa-Năsăud County: (LN 36) Rodnei Mountains National Park, Cascada Cailor, lat. 47.5951°N, long. 24.797°E, alt. 1207 m, Rotunda Pass, lat. 47.5244°N, long. 24.9998°E, alt. 1044 m, Repedea Valley, lat. 47.5943°N, long. 24.6815°E, alt. 1180 m, Borşa 1, lat. 47.6224°N, long. 24.6396°E, alt. 1048 m, Borşa 2, lat. 47.6504°N, long. 24.6356°E, alt. 783 m, Izvorul Dragoş Valley, lat. 47.6211°N, long, 24.6125°E, alt. 957 m (Ardelean *et al*. 2013).

6. *Parmelia sulcata* Taylor 1836

Alba County: (FR 87) Donea Forest, Donea Hill (Moruzi *et al*. 1967; Ciurchea, 2004); (KM71) Hususău Forest (Cretzoiu, 1939; Moruzi *et al*. 1967; Ciurchea, 2004); (GS 03/13) near Aiud town (Moruzi *et al*. 1967; Ciurchea, 2004); (GS 03) Bichiş, Rărişti, Lopadea, Vălişoara forests, in the neighbourhoods of Aiud (Györkös and Bartók, 2006); (FS 62/63) Roşia Montană (Crişan and Ardelean, 2010).

Arad County: Apuseni Mountains, Zarandului Mountains (ES 91) Slatina de Mureş; (ES 60) Milova; (ES 50) Cladova (Bartók, 1994; Ciurchea, 2004).

Argeş County: (LK 18) near Lintești locality, lat. 44.99625°N, long. 24.62831°E, alt. 519 m, leg. Vicol Ioana, 06.10.2015, det. Vicol Ioana, 10.12.2015, [BUCM L2778].

Bacău County: (MM 95/96) Bacău, leg. Maria Gabriel Mihai, 07.09.2015, det. Vicol Ioana, 19.10.2015, [BUCM L2718].

Bihor County: (FS 17) Bihorului Mountains, Stâna de Vale (Borza and Ţenchea 1946; Moruzi *et al*. 1967; Ciurchea, 2004); (FS 28) Valea Bulzului (Bartók, 1981; Ciurchea, 2004); (ES 59) Rădvani Forest near Salonta (Pop, 1962; Moruzi *et al*. 1967; Ciurchea, 2004); (FS 27) Bihorului Mountains on the Răchita Peak (Bartók 1982); (ET 61/70/71) Pădurea Craiului Mountains (Crişan, 2003).

Bistriţa-Năsăud County: (LN 14/16) Rodnei Mountains, Rebra Valley (Bartók, 1988; Ciurchea, 2004); (LN 36) Rodnei Mountains National Park, Cascada Cailor, lat. 47.5951°N, long. 24.797°E, alt. 1207 m, Rotunda Pass, lat. 47.5284°N, long. 25.0041°E, alt. 1089 m, Repedea Valley, lat. 47.5943°N, long. 24.6815°E, alt. 1180 m, Borşa 1, lat. 47.6224°N, long. 24.6396°E, alt. 1048 m, Borşa 1, lat. 47.6287°N, long. 24.6635°E, alt. 1988, Izvorul Dragoş Valley, lat. 47.6209°N, long. 24.6176°E, alt. 1036 m, Pietrosul Mare, lat. 47.6053°N, long. 24.6026°E, alt. 1470 m (Ardelean *et al*. 2013); (KN 91) forests around Arcalia Scientific Stationary (Ciurchea, 1972; Ciurchea, 2004); (LN 27) Năsăudului hills,

Satului Valley (Bartók, 1987a; Ciurchea, 2004); (LN 03/04) between Bistrița and Rebrișoara localities, leg. Dumitrescu Florentina, 07.10.2015, det. Vicol Ioana, 19.10.2015, [BUCM L2701]; (KN 92) Șintereag, leg. Dumitrescu Florentina, 06.10.2015, det. Vicol Ioana, 19.10.2015, [BUCM L2709].

Botoșani County: (MP 41) Pădureni Forest (Burlacu, 1967; Ciurchea, 2004); (MP 50) Horlăceni and Gorovei forests (Burlacu, 1969b; Ciurchea, 2004).

Bucharest: (MK 21/22/31/32) Băneasa Forest, leg. Vicol Ioana, 25.03.2011, det. Vicol Ioana, 13.05.2011, [BUCM L1644].

Buzău County: (ML 63) Trestioara Forest, (ML 42) Ruginoasa Forest near Mlăjet (Moruzi and Mantu, 1962; Ciurchea, 2004).

Caraș-Severin County: Cernei Mountains, (FQ 05/15) Cernei Valley at Băile Herculane; (FQ 16/17) Domogled Mountain; (EQ 45) Glavcina Hill; (EQ 55) Țigansca Reca (Moruzi *et al*. 1967; Ciurchea, 2004); Danube Defile (EQ 84) Cozla, (EQ 54) Pescari (Burlacu *et al*. 1969; Ciurchea, 2004); (EQ 98) between Borlovenii Noi and Globu Craiovei, lat. 44.99771°N, long. 22.17660°E, leg. Vicol Ioan, 17.09.2015, det. Vicol Ioana, 19.10.2015, [BUCM L2697].

Călărași County: (MK 62) Călăreților Forest, leg. Vicol Ioan, 04.06.2010, [BUCM L1303], (MK 62) Goștilele Forest, leg. Vicol Ioana, 30.06.2010, [BUCM L1466], Vicol (2011b).

Cluj County: (FS 38/48) Vlădeasa Mountain, Răcad Valley (Bartók, 1988; Ciurchea, 2004); (GS 15/16) Băile Sărate, Turda (Todor, 1947; Ciurchea, 2004); (FS 97/98/GS08) near Cluj-Napoca, in orchards (Bartók, 1999), Cluj-Napoca in Botanical Gardens (Codoreanu *et al*. 1960; Moruzi *et al*. 1967; Ciurchea, 2004); (FS 97/GS07) Feleacu, in orchards (Bartók, 1999); (FS 87/88) Râșca Valley (Crișan and Tegzeș, 2006); (FS 86) Bocului Mountain, at Roșala, Dealul Huzii, Boc hamlet (Tothăzan and Crișan, 2008).

Dâmbovița County: (LL 51) Leaota Mountains, Râiosul and Romanescu Mountains, Brăteiului Valley (Burlacu and Diaconescu, 1969; Ciurchea, 2004).

Giurgiu County: (MJ 39) Comana Forest (Moruzi *et al*. 1967; Ciurchea, 2004); (LK 92) Căsciorelor Forest (Vicol, 2011b).

Gorj County: (GR 02) Parâng Mountain, Slăveiul Mare Peak (Ciurchea, 2004).

Harghita County: (LN 80/90) near Borsec locality, lat. 46.96561°N, long. 25.58633°E, alt. 790 m, leg. Vicol Ioana, 17.09.2015, det. Vicol Ioana, 19.10.2015, [BUCM L2700].

Hunedoara County: (FR 42) Retezat Mountains, Slăveiul Mountain, Râu de Mori, Râușor Valley (Zschacke, 1913; Moruzi et Mantu, 1962; Ciurchea, 2004); Râul Mare Valley (Szatala, 1932; Moruzi *et al*. 1967), towards Bucura Lake, Câmpușel, Scorota cu apă, Retezat National Park (Moruzi and Mantu, 1962; Ciurchea, 2004); (FR 85) Șureanu Mountains, Ausșelul Mountain (Moruzi and Mantu, 1962; Ciurchea, 2004); (FR 65) Poiana Ruscăi Mountains, Bega Luncani (Bartók *et al*. 2005).

Iași County: (MN 95) Dealul Mare-Hârlău (Burlacu, 1969c; Ciurchea, 2004).

Ilfov County: (MK 13) Mogoșoaia Forest (Moruzi and Mantu, 1965); (MK 13) Buftea Forest; (MK 34/35) Snagov Forest; (MK 42) Pustnicul Forest (Moruzi *et al*. 1967; Ciurchea, 2004); (MK 32) Andronache Forest, leg. Vicol Ioan, 24.03.2009, det. Vicol Ioana, 30.06.2009, [BUCM L1302], (MK 42) Pustnicul Forest, leg. Vicol Ioan, 30.04.2009, det. Vicol Ioana, 23.06.2009, [BUCM L1352], leg. Vicol Ioan, 27.03.2009, det. Vicol Ioana, 22.06.2009, [BUCM L1353], Vicol (2010); (MK 34/35) Snagov Forest, leg. Vicol Ioana, 08.06.2010, [BUCM L1304], (MK 24) Vlădiceasca Forest, leg. Vicol Ioana, 26.04.2010, det. 11.05.2010, [BUCM L1354], leg. 06.06.2010, [BUCM L1337], (Mantu, 1965; Vicol, 2011b); (MK 34) Biglaru Forest, leg. Vicol Ioana, 27.04.2010 [BUCM L1355], Vicol (2011c); (MK 42) Pustnicul Forest, leg. Vicol Ioana, 26.08.2011, det. Vicol Ioana, 02.09.2011, [BUCM L1647].

Maramureș County: (GU 20) near Fereşti locality, lat. 47.82584°N, long. 23.93940°E, alt. 364 m, leg. Vicol Ioan, 15.11.2015, det. Vicol Ioana, 15.11.2015, [BUCM L2769].

Mehedinți County: (FQ 38/48) Gorjului Subcarpathians at Plaiul Ponoarele; (FQ 55) Pârâul Peșteana; (FQ 05/15) Danube Defile at Orșova (Moruzi *et al*. 1967; Ciurchea, 2004).

Mureș County: (LM 37) Sân-Mihai de Pădure (Moruzi and Mantu, 1962; Ciurchea, 2004); (LN 50) Bistrei Valley at Zăpodea cu Calea and Gălăoaia Rivulet, Landscape Reserve Defileul Deda-Toplița (Crișan and Florea, 2005), Neagră Brook, Landscape Reserve Defileul Deda-Toplița (Crișan and Banc, 2007).

Neamț County: (MN 43) Mănăstirea Neamț; (MN 39) Tarcăului Mountains; (MM 18) Cheile Bicazului (Moruzi *et al*. 1967; Burlacu, 1969a; Ciurchea, 2004); (MN 20) Ceahlău Mountain at Piciorul Maicilor, Ciribuc Glade, Faloni Glade, Piciorul Bucura, Negrei Valley, Izvorului Alb Valley, Bâtca Durăului, Izvorul Muntelui and Horștei Peak (Manoliu *et al*. 1998); (MN 52) Humăria Forest (Ciurchea, 2004).

Prahova County: (LL 91) Baiului Mountains, Cumpătul Mountains, leg. Pîrsoagă Alina, 08.10.1983, det. Zamfir Manuela, 08.10.1983, [0178]; (LL 73) Bucegi Mountains, Poiana Coștilei (Moruzi and Mantu, 1962; Ciurchea, 2004); (LL73) Bucegi Mountains, Furnica Mountain (LL 81/82) Sinaia (Zamfir *et al*. 1998); (ML 13) Ciucaș Mountains, Muntele Babeșu (Mantu, 1967; Moruzi and Mantu, 1962; Ciurchea, 2004), (LL 91/ML 01) Glodeasa Nature Reserve, leg. 13.05.2009, det. Vicol Ioana, 19.07.2009, [BUCM L1418], Vicol (2011a).

Sălaj County: (FT 70) near Sutor locality, leg. Vicol Ioana, 02.09.2015, det. Vicol Ioana, 28.09.2015, [BUCM L2662], lat. 46.96141°N, long. 23.31726°E, alt. 334 m, leg. Vicol Ioana, 02.09.2015, det. Vicol Ioana, 10.12.2015, [BUCM L2774]; (FT 62) Ciumârâna, leg. Dumitrescu Florentina, 25.08.2015, det. Vicol Ioana, 28.09.2015, [BUCM L2677].

Sibiu County: (LL 15) Făgăraș Mountains, Suru Peak and Arpașul Mare Mountain (Moruzi *et al.* 1967; Ciurchea, 2004); (LL 17) Tătarilor Lake Nature Reserve, leg. Vicol Ioana, 29.03.2011, det. Vicol Ioana, 31.03.2011, [BUCM L1639]; (KL 85/86) Tălmaciu Forest, leg. Vicol Ioana, 28.03.2011, det. Vicol Ioana, 05.04.2011 [BUCM L1641].

Suceava County: (LN 95) Pietrosul Broștenilor Mountain, Piciorul Călugărului, Pârâul Rânculeț (Moruzi and Mantu, 1962; Ciurchea, 2004); (LN 95) Rarău Mountain (Moruzi and Mantu, 1962; Burlacu, 1969c; Ciurchea, 2004); (LN 84) Bistriței Mountains, Pârâul Caprei, Pârâul Vacariei, Cozănești, Barnar Valley (Popa, 2006); Codrul Secular Slătioara Forest Reserve, leg. Vicol Ioana, 26.06.2013, det. Vicol Ioana, 08.07.2013, [BUCM L2051]; (MN 54/55) Manolea Forest (Moruzi and Mantu, 1962; Ciurchea, 2004); (LN 84) Bistriței Mountains, Rusca locality, leg. Vicol Ioana, 10.03.2015, det. Vicol Ioana, 22.04.2015, [BUCM L2622]; (LN 54/64) Poiana Stampei, lat. 47.30477°N, long. 25.11744°E, alt. 935 m, leg. Vicol Ioana, 17.09.2015, det. Vicol Ioana, 28.09.2015, [BUCM L2690].

Tulcea County: (PL 91) Letea Forest, Haşmacul Uje (Moruzi *et al.* 1967; Mantu, 1968; Ciurchea, 2004); (PK 18) Horia (Yavuz and Çobanoğlu, 2008); (PK 89) Caraorman Grind, Danube Delta (Toma and Covaliuc, 2006); (NL 60) Măcin Mountains, leg. Vicol Ioana, 22.05.2013, det. Vicol Ioana, 27.05.2013 [BUCM L1928], [BUCM L2012]; lat. 45°09’23’’N, long. 28°18’42’’E, alt. 264 m, on saxicolous substrata, leg. Vicol Ioana, 22.05.2013, det. Vicol Ioana, 03.06.2013, [BUCM L1970].

Vaslui County: (NM 40) Tutovei forests, Gândeasa Forest (NM 55) Lipovăț Forest, Căpușneni Forest (Moruzi and Mantu, 1962; Ciurchea, 2004); (NM 46) Bălteni Forest Natural Reserve, leg. Vicol Ioana, 17.08.2012, det. Vicol Ioana, 11.09.2012 [BUCM L1837].

Vâlcea County: (KL 92) Cozia Mountain (Bartók, 1990; Ciurchea, 2004); (KL 92) Cozia National Park (Çobanoğlu et al. 2009).

Vrancea County: (ML 79) Cârligata Forest (Burlacu, 1969c; Ciurchea, 2004).

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COMPARISON OF MORPHOLOGICAL TRAITS CHANGES IN PROLONGED VEGETATIVE REPRODUCTION OF THREE CROP FLAX (*LINUM USITATISSIMUM* L.) POPULATIONS AND WILD (*LINUM ALBUM*) DOMESTICATED IN IRAN

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Somaclonal variation of four flax populations, namely 'Saveh', 'Uromieh', 'Shiraz' and 'Fars, during long-term propagation were evaluated among 3 subcultures. Morphological traits such as length of shoot, number of shoot, number of bud, length and number of root and number of leaves were measured. Our results showed that flax (*Linum usitatissimum* L.) Saveh, Uromieh and Shiraz populations more than (*Linum album*) Fars population affected somaclonal variations especially regeneration plantlets (for example: length of stem and number of stems, buds and leaves) but Fars population had steady behavior especially length of shoot and number of shoot during several subcultures. Although in all traits fluctuating changes were observed but the most significant traits studied with almost similar vibration in four populations were number of nodes and leaves values. Totally we could not select a specific subculture period for creation of the maximum satisfied morphological changes because it was a suitable increase of leaf and node number in Fars and Saveh populations in the first subculture and it was suitable for Uromieh and Shiraz populations in the second subculture. In order to accomplish the morphological changes in length of shoots, number of stems and enhancement of node in (*Linum usitatissimum* L.) three of populations and also decrease of number of leaves in (*Linum album*) Fars population somaclonal variation during three subcultures will be appropriate.

Keywords: Population, Regenerated plantlet, Somaclonal variation.

Abbreviations: MS: Murashige & Skoog, BA:6- Benzyl amino purine, NAA: Naphthol acetic acid, pop. Population.

INTRODUCTION

Linum is the largest genus of *Linaceae*, with about 230 species (Heywood 1993). These species mainly grow in temperate and subtropical regions of the world (Rogers, 1982; Muir & Westcott, 2003). Linseed (*Linum usitatissimum* L.) is one of the oldest cultivated plants and is the only member of *Linaceae* that is economic important. Members of this genus grow naturally in Iran. So far 22

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species and infra specific taxa have been reported from Iran classified in five sections. *Linum album* Ky ex Boiss. is one of them and belongs to the section *Syllinum* Griseb. This species is endemic to Iran and is considered an Irano-Turanian element naturally widespread in different regions of the northern, northwestern, western and central parts of Iran (Rechinger, 1974; Sharifnia & Assadi, 2001).

In the world production of textile fabrics, priority is given to flax fiber showing higher medicobiological and physicomechanical properties than cotton fiber (**Pretová** *et al.*, 2001; Pretová & Obert, 2001). Demand for flax and flax containing textile fabrics increases from year to year owing to such a unique set of flax properties as hygienic ability, high strength, low electric resistance, dust capacity, comfort ability, and natural bactericidal action (antiseptic and antiputrefactive action) (Guzenko *et al.*, 2009).

Seeds of oil-yielding flax are capable of accumulating from 35 to 52% of oil. A high content of linolenic acid makes it a good dehydrator and allows its application in production of paints, varnishes, drying oil and roadway covering (Green & Marshall, 1984), while lignans have been identified in flowering aerial parts of these species (Schmidt *et al.*, 2010).

Development and introduction of advanced technologies for complex processing of flax seed, use in animal feed, as well as for dietary and medical purposes have led to the fact that some countries growing mainly spinning flax began to carry out scientific programs for studying linseed gene pool (Pavelek *et al.*, 1996).

Extensive research has been done to conserve and explore germplasm of crop wild relatives that are closely related to the cultivated crops and may contain beneficial traits (Dwivedi *et al.*, 2008).

Commonly, flax breeders use pedigree selection or bulk breeding methods to create novel lines (Steiss *et al.*, 1996). The application of biotechnology has been helpful in accelerating breeding programs or improving the efficiency of selection, as demonstrated in linseed flax and other oil crop species (Friedt, 1990). Tissue culture techniques developed for flax mainly aim to obtain valuable and desirable new traits in flax cultivars (resistance to fungal diseases, oil quality improvement and herbicide tolerance) through somatic hybridization and somaclonal variation (Basiran *et al.*, 1987). Cotyledons, hypocotyls, meristems, and stem segments have been used as explants for culture initiation in flax, but only plant regeneration from hypocotyl segments has proven to be highly efficient (Friedt, 1990). The aim of this research for the first time was to determine the effect of genotype and medium composition on *Linum usitatissimum* L. and *Linum album* morphogenesis *in vitro*.

MATERIALS AND METHODS

The investigation was carried out with three linseed crop flax (*Linum usitatissimum* L.) populations 'Saveh', 'Uromieh', 'Shiraz' and wild flax (*Linum album*) population 'Fars'. Plump and smooth flax seeds were selected. The flax seeds were

soaked in a 70% alcohol solution for 1 minute and then transferred into 40% bleach solution, which was leached by sterile filter, then washed three times with sterile distilled water. Sterilized seeds were germinated on agar (8 g/L) at 24ºC and dark condition for 5-7 days (Fig.1a, Fig.1b). Stem segments and hypocotyls from seedlings were used as explants to study flax morphogenesis. Tissue culture protocols were the same as described previously (Bretagne *et al.*, 1994; Blinstrubiene *et al.*, 2004). Explants were placed on medium MS salts (Murashige & Skoog, 1962) with sucrose 30 g/L and agar 6 g/L and the following growth regulators were used according to Table 1. The media pH was 5.7, illumination – 5000 lx, photoperiod-16 h, temperature 25± 2°C. Each variant consisted of 50 explants and four replications were used. The best medium culture supplemented hormones selected and single node of plantlets cultured on B medium (MS basal medium with BA, 0.5 mg/L and NAA, 1.5 mg/L). Explants were transferred to 50 mL fresh medium every 6 weeks into 200 mL glassware. The regeneration potential of tissues was evaluated by analyzing the morphological parameters of the structures formed in the explants during three subcultures. The evaluation was based on the relative frequency of explants forming length of shoots, number of shoots, number of bud/shoot, number of roots, length of roots and number of leaves. Mother plants comprised regenerated flax plantlets during subcultures. Significant differences were determined using a computer programme SPSS (Ver. 22) for analysis of variance, grouped by Duncan's criteria $P \leq 0.05$.

Table 1

A kind of medium culture used for regeneration plantlets

Medium	BA (mg/L)	NAA (mg/L)
A	0.5	0.5
B	0.5	1.5
C	1.5	0.5
D	1.5	1.5

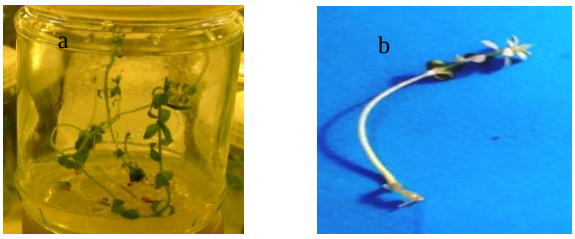

Figure 1. *In vitro* seedling growth from a) *Linum usitatissimum* and b) *Linum album* seeds.

RESULTS AND DISCUSSION

Regeneration capacity of plants shows a wide range among families, species and even within genotypes from the same species. The composition of growth medium is an important factor affecting growth and morphogenesis of

plant tissues. Plant tissue culture medium consists of macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, carbon sources, organic supplements, solidifying agents and growth regulators. Murashige and Skoog (1962) are the most commonly used medium in plant tissue culture. When comparing the studied media (Table 1), B medium (MS basal medium with BA, 0.5 mg/L and NAA, 1.5 mg/L) gave the highest average number of buds per hypocotyl across all populations, while other media ranked lowest. The B medium was the best medium for regeneration plantlets and it was selected for other experiments. Linseed flax morphogenesis in tissue culture is influenced by endogenous and exogenous factors: pop. 'Uromieh' had the highest frequency of shoot formation in B medium. B nutrient medium supplemented with cytokinin BA (0.5 mg/L) and auxin NAA (1.5 mg/L) was the mostly suitable medium for linseed flax organogenesis *in vitro*.

We have chosen shoot segments as explants for producing cell and tissue culture of the analyzed populations as a number of research works (Bretagne *et al.*, 1994; Dedicova *et al.*, 2000) show that the given explant type is the most suitable for successful initiation of callus, organogenesis and embryogenesis in *Linum usitatissimum*. Regeneration processes in shoot segments of the populations under study began, on the average, on the second weeks of culturing (shoot initiation points emerged along the length of explant). Shoot regeneration was observed from stem segment explants, but with higher incidence near the cut ends. This finding agrees with that of Gamborg and Shyluk (1976); they suggested this was due to an inherent hormonal gradient within the hypocotyl tissue cells. In the present study, many buds arose directly from the surface of the explants without forming any callus. The analysis results of the obtained data on the morphogenetic potential and regenerative ability depending on populations. The degree of regeneration response, expressed as number of differentiated buds per shoot, varied among the populations and among the number of subcultures examined, and these variations were significant at 0.05 level of probability. Also, the variation due to populations x number of subculture was highly significant. Among the three studied populations of flax (*Linum usitatissimum*), regenerated Uromieh pop. plantlet showed the greatest length of shoot (5 cm) and number of shoot (1) and number of differentiated bud per shoot (about 6) and number of leaves (21) followed by regenerated Shiraz pop. plantlet (4 cm length of shoot and 4 number of bud) and regenerated Saveh pop. plantlet (4 cm length of shoot and 3 number of bud), while the regenerated Fars pop. plantlet (*Linum album*) ranked with mean of length shoot (2 cm), mean of number shoot (6) and an average of 10 buds/shoot (Fig. 2). The difference between Uromieh pop. and Shiraz pop., however, was not significant at 0.05 level of probability, likely due to the close relationship of these two populations as they share a common parent (Koronfel, 1994).

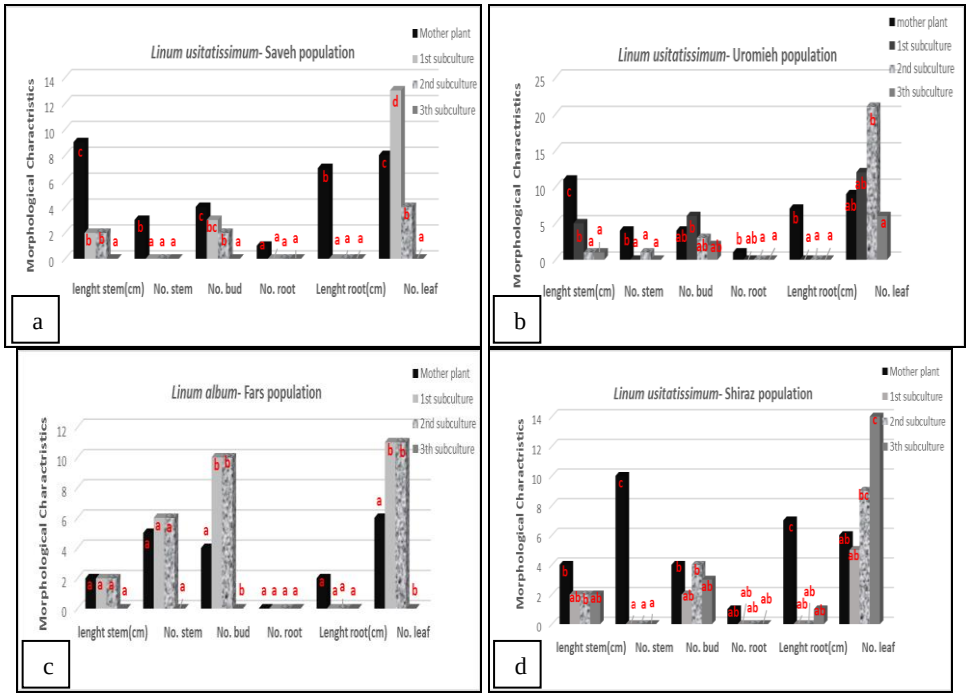

Figure 2. Comparison of mother plants to regenerated plantlets during subcultures in *Linum usitatissimum* L. populations a) 'Saveh' b) 'Uromieh', c) 'Shiraz' and d) *Linum album* Fars population.

The influence of population on the ability of plants to regenerate *in vitro* was *previously* pointed out (Tomes & Smith, 1985; Zhan *et al.*, 1989a; Koronfel, 1994). Duncan *et al.*, (1985) have shown that the poor response of some genotypes can be compensated to some extent by varying the culture conditions of either the source material or of the explants. When comparing populations in the studied medium culture (0.5 mg/L BA, 1.5 mg/L NAA), Fars pop. plantlet (*Linum album*) gave the highest average number of buds per shoot (10 buds), while regenerated Saveh pop. plantlet ranked lowest (2 buds).The superiority of medium culture over other studied media was obviously not only by increasing the frequency of sites of bud initiation, but also by promoting the formation of clusters of buds at these sites. This may be attributed to the addition of a cytokinin (BA) as a sole phytohormone (with auxin added to this medium). Auxins have commonly been reported to be either inhibitory or neutral for shoot regeneration (Kartha *et al.*, 1974; Fonnesbech *et al.*, 1977; Lane, 1979; Zhan *et al.*, 1989a) but are sometimes stimulatory (Miller & Murashige, 1976). In the present study, in comparison with hormone-free medium, the addition of cytokinin (BA) in combination with auxin (NAA) stimulated shoot bud formation. In this respect, our results confirm the previous ones reported by Lane (1979) and Zhan *et al.*, (1989b). Direct bud regeneration occurred, in this study, very rapidly and efficiently on the surface of shoot segment explants, in a manner similar to that reported for *Linum*

marginale, in which buds arise directly from epidermal cells of the shoot (Zhan *et al.*, 1989a). This kind of regeneration might be useful for genetic transformation techniques. The phenomenon of direct bud formation from shoot segments is rare in most plant species, butit occurs in *Linum usitatissimum* and *Linum album*. Link and Eggers (1946) and Zhan *et al.*, (1989b) reported directly bud formation from shoots in two species of the genus *Linum*. All shoot initials tested for rooting did not develop normal roots when transferred to medium with hormone during 3 subcultures. Investigating morphological characteristics was studied in *Linum usitatissimum* (Saveh, Uromieh and Shiraz populations) and *Linum album* (Fars population) during three subcultures. In Saveh pop. the largest mean of length shoot regenerated plantlet (2 cm), number of buds (node) (3) and number of leaves (13) were observed in the first subculture, data showing a significant difference to mother plants ($p \leq 0.05$) (Fig. 3).

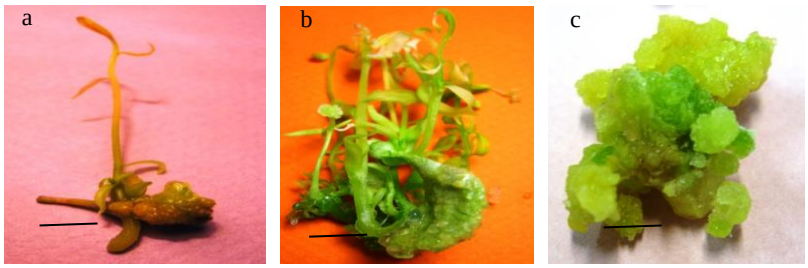

Figure 3. Adventitious shoot regeneration from shoot explants of flax (*Linum usitatissimum* L.) populations 'Saveh' a) 1st subculture, b) 2nd subculture and c) 3th subculture interval 4 weeks (error bar: 1cm)

In Uromieh pop., the highest mean of number shoot regenerated plantlet (1), number of buds (3) and number of leaves (21) were shown in the second subculture and the maximum mean of length shoot (5) was in the first subculture, the number of leaves showed no significant difference to mother plants ($p \leq 0.05$) (Fig. 4).

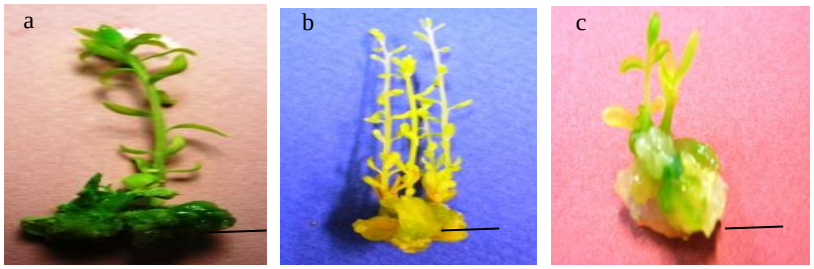

Figure 4. Adventitious shoot regeneration from shoot explants of flax (*Linum usitatissimum* L.) populations 'Uromieh' a)1st subculture, b)2nd subculture and c)3rd subculture interval 4 weeks (error bar: 1cm)

In Shiraz pop., the largest mean of length shoot regenerated plantlet (4 cm), number of buds (4) and the number of leaves (9) were in the second subculture, the number of leaves showing no significant difference to mother plants ($p \leq 0.05$) (Fig. 5).

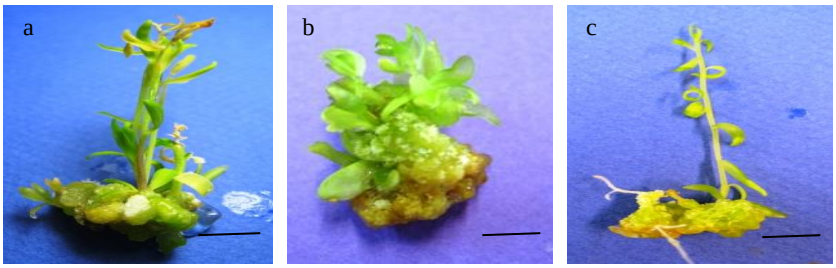

Figure 5. Adventitious shoot regeneration from shoot explants of flax (*Linum usitatissimum* L.) populations 'Shiraz' a)1st subculture, b)2nd subculture and c)3rd subculture interval 4 weeks (error bar: 1cm)

In Fars pop., the mean of length shoot regenerated plantlets (2 cm), the number of shoots (6), number of buds (10) and number of leaves (11) were observed during two subcultures, there was a significant difference to mother plants ($p \leq 0.05$) (Fig. 6).

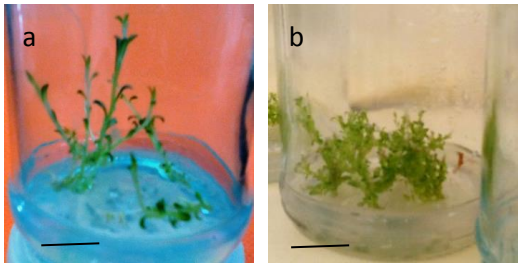

Figure 6. Adventitious shoot regeneration from shoot explants of flax (*Linum album*) population 'Fars' a)1st subculture and b)2nd subculture interval 4 weeks (error bar: 1cm)

Morphogenesis is strongly affected by genetic and exogenous factors (Bhaskaran & Smith, 1990; Bjowani & Razdan, 1990). Our results illustrate that the genetic background is important both for callus induction and shoot regeneration in linseed flax tissue culture. The linseed flax pop. 'Uromieh' showed the superior morphogenetic capability. The organogenesis capacity was higher in cv. 'Uoromieh' by 1.5 and 1.1 times compared to pops. 'Shiraz' and 'Saveh', respectively and pop. Fars was higher 2 times compared to pop. Uromieh. Depending on the plant species, nutrient media are often modified by adding different compositions of vitamins and growth regulators. The most widely used growth regulators are the cytokinins BAP, 2iP and kinetin (Bjowani & Razdan,

1990), and the auxins IAA and NAA. The effect of medium composition on linseed flax callogenesis, shoot formation and rhizogenesis strongly depended on the population and the type of explant, with the four tested linseed flax cultivars exhibiting different regeneration responses. Shoot segments from the linseed flax cultivar 'Uromieh' gave the best results. It can be assumed that the differences in morphogenetic reaction of different linseed flax populations are determined by the balance of endogenous hormones. The combinations of growth regulators optimal for callus induction, root formation and shoot regeneration differed.

Our results show that linseed morphogenesis capacity depends not only on growth regulators, but also on the other components (macro salts, micro salts, vitamins). The best medium (MS + 0.5 mg/l BA, 1.5 mg/l NAA) differed from the others in raised quantities of added vitamins and amino acids, which allowed more intensive development of callusogenesis and organogenesis in the linseed flax tissue culture.

CONCLUSION

The described tissue culture procedure in this study could be used for the vegetative propagation or cloning of a particular line of flax; it might also be useful in facilitating genetic transformation attempts.

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THE INTERACTIVE EFFECTS OF CADMIUM AND GA_3 ON TOMATO (*LYCOPERSICON ESCULENTUM* MILL. CV. CH) PLANTS PHOTOSYNTHESIS, ANTHOCYANIN, PROLINE AND TOTAL PHENOLIC CONTENTS

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The individual and interactive effects of different Cd and GA_3 concentrations were investigated in tomato (*Lycopersicon esculentum* Mill. cv. CH) plants grown under greenhouse conditions. Treatments were prepared by adding 0, 2.5, 5, 10 and 20 µM Cd and 0, 5 and 10 µM GA_3 to the Hoagland nutrient solution. Exposure to different Cd concentrations led to decrease relative water content, chlorophylls *a* and *b*, carotenoids, total proteins contents and net photosynthetic rate. In contrast, respiration rate, CO_2 compensation concentration, proline, anthocyanin and total phenolic contents enhanced along with Cd concentration increase. On the other side, GA_3 treatments led to increase net photosynthetic rate, relative water content, proline, chlorophylls *a* and *b*, carotenoids, anthocyanin, total phenolic and total proteins contents, while, the respiration rate and CO_2 compensation concentration decreased. Two-way ANOVA analysis showed that GA_3 treatments overcame the toxic impacts of Cd and promoted plant tolerance against its toxicity.

Keywords*:* Photosynthesis, Metal toxicity, Total phenolic, Protein, Anthocyanin.

Abbreviation*:* Cadmium (Cd); Gibberellic acid 3 (GA_3); Relative water content (RWC); Reactive oxygen species (ROS); δ-aminolavulinic acid (ALA).

INTRODUCTION

Cadmium is an extremely toxic heavy metal for animals and plants with a long biological half-life (Wang *et al.* 2008). The main sources of Cd pollutions are volcanic eruptions, mining, sewage sludge, phosphate fertilizers and urban traffics (Benavides *et al.* 2005; Gill and Tuteja, 2010). The plants easily uptake Cd via divalent cations transporters and accumulate it in their different parts such as roots, shoots, fruits and grains (Lopez-Millan *et al.* 2009). Therefore, Cd toxicity has become a serious problem for human and animal health (Qian *et al.* 2009).

Cd, as a toxic element, adversely impacts some of plants' processes at genetical, biochemical and physiological levels (Nedjimi and Daoud, 2009). Several recent studies described that some of Cd-induced physiological changes include interruption of water balance, reduction of photosynthetic yield, oxidative

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stress, disturbance in plant mineral nutrients uptake and distribution (Gill and Tuteja, 2010; Hall, 2002), interaction with nucleic acids and disruption of photosynthetic electron transport (Lopez-Millan *et al.* 2009).

Plants for detoxification of heavy metals effects have diverse mechanisms at cellular and molecular levels (Hall, 2002). The first barrier against Cd toxicity located at roots which Cd immobilized by binding to cell wall and extra cellular carbohydrates such as mucilage and callose (Benavides *et al.* 2005). Within the cytosol Cd is chelated with different components such as phytochelatins, organic acids, amino acids and sequestrated into vacuole (Hall, 2002).

The protective roles of GA_3 against some of stress conditions have been reported (Tuna *et al.* 2008). There are some works that reported alleviative effects of GA_3 on heavy metal toxicity and salinity in some of plants such as *Glycine max* (Saeidi-Sar *et al.* 2007) and *Triticum aestivum* (Siddiqui *et al.* 2011).

The purpose of this study was to examine involvement of GA_3 in Cd toxicity amelioration in tomato plants by determination of some of physiological changes such as proline, anthocyanin, total phenolic and photosynthetic pigments contents.

MATERIALS AND METHODS

Plant materials, growth conditions and stress treatments

The seeds of tomato (*Lycopersicon esculentum* Mill. cv. CH) plants were supplied by Falat Company, Tehran, Iran. Before sowing, tomato seeds were sterilized with 1% sodium hypochlorite for 1 min, then they were rinsed with distilled water and germinated in petri dishes containing moist filter-paper. Afterwards, they were transferred to pots containing sterilized moist sand and placed in greenhouse with natural light (16-8 h photoperiod), temperature (25 ± 1°C on day and 18 ± 1°C at night), light intensity about 60 µmol m^{-2} s^{-1} and irrigated with Hoagland's nutrient solution. Then, 30-day-old plants were treated with different concentrations of $CdCl_2$ (0, 2.5, 5, 10 and 20 µM) and GA_3 (0, 5 and 10 µM). Finally, 60-day-old plants were harvested and used for determination of physiological parameters.

Gas exchange analysis

Net photosynthetic rate, respiration rate and CO_2 compensation concentration were determined based on the method of Khavari-Nejad (1980, 1986) in plant leaves by using an infrared gas (CO_2) analyser (ADC-225-MK3) apparatus.

Leaf relative water content (RWC) determination

Leaf relative water content (RWC) was measured according to the method of Weatherley (1950) by using the equation below:

$$\text{RWC}\ (\%) = [\frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}] \times 100$$

where DM, FM, and TM are the dry, fresh and turgid masses, respectively, of the tissue.

Photosynthetic pigments determination

The contents of chlorophylls *a*, *b* and carotenoids of samples were measured spectrophotometrically according to the method of Lichtenthaler (1987).

Total protein assay

The content of total protein was measured according to the method of Bradford (1976) by using bovine serum albumin (BSA) as standard.

Proline content assay

The proline content was determined according to the method described by Bates *et al*. (1973) and the content of proline was expressed as a $\mu g\ g^{-1}$ F.W.

Anthocyanin and total phenolic content determination

The contents of anthocyanin and total phenolic were determined according to the method of Dai *et al.* (2006). The leaves (0.1 g) were homogenized and extracted with 1% HCl-methanol and the resulted extracts were placed in darkness at 4 °C. After 24 h, extracts were centrifuged at 4000 g for 10 min. The absorbance of solution was measured at 600 and 530 nm for estimation of anthocyanin contents and 280 nm for determination of total phenolic contents.

Statistical analyses

The data are means of four replications with standard error of mean (SEM). All statistical analyses (two-way analysis of variance (ANOVA)) were performed with Duncan's test by using SPSS 17 and SAS 9 statistical softwares at the $P < 0.05$ level of probability.

RESULTS AND DISCUSSION

Effects of Cd and GA_3 on gas exchange

The resulted data from effects of different concentrations of Cd and GA_3 on gas exchanges were shown in Table 1. It is well-known that photosynthetic organisms are extremely sensitive to heavy metal toxicity. Cd is a non-essential metal and it can decrease the activity of photosystem II (PSII) (Qian *et al.* 2009). It has been proved that Cd disturbs the electron transport chain and alters genes expression involving in synthesis of PSII proteins (Benavides *et al.* 2005). Decline in photosynthetic rate could be partially explained by the competition between Cd and Mn uptake and transport which Mn is necessary for optimal activity of water-splitting complex (Lopez-Millan *et al.* 2009). Besides, depression photosynthesis may be due to inhibition of the activities of some enzymes involved in photosynthetic carbon reduction cycle. For example, the inhibitory effect of Cd on activities of 3-phosphoglyceric acid kinase and rubisco has been reported (Qian *et al.* 2009).

On the other side, the application of Cd led to increased respiration rate and CO_2 compensation concentration (Table 1) which coordinated with decline in photosynthetic rate. Under Cd stress, increased oxidative metabolism and activities of some enzymes from the Krebs cycle such as malate dehydrogenase, citrate synthase and isocitrate dehydrogenase have been reported (Lopez-Millan *et al.* 2009).

Table 1

The effects of GA_3 application on the net photosynthetic rate, respiration rate and CO_2 compensation concentration of Cd stressed plants

$CdCl_2$ (µM)	GA_3 (µM)	Net photosynthetic rate (µmol CO_2 m^{-2} s^{-1})	Respiration rate (µmol CO_2 m^{-2} s^{-1})	CO_2 compensation Concentration (µl l^{-1})
0	0	1.58±0.0040^{c}	0.22±0.0047^{gh}	54.72±0.60^{h}
	5	1.86±0.0075^{b}	0.16±0.0028^{j}	45.27±0.57^{i}
	10	2.01±0.0070^{a}	0.09±0.0040^{k}	42.63±0.53^{j}
2.5	0	1.03±0.0028^{i}	0.26±.0040^{e}	62.79±0.64^{f}
	5	1.15±0.0064^{f}	0.22±0.0050^{ih}	58.32±0.51^{g}
	10	1.36±0.0086^{d}	0.21±0.0025^{i}	54.16±0.59^{h}
5	0	1.16±0.0025^{f}	0.33±0.0028^{b}	73.50±0.54^{c}
	5	1.29±0.0085^{e}	0.23±0.0025^{gf}	69.40±0.62^{d}
	10	1.31±0.0064^{e}	0.29±0.0086^{c}	54.18±0.61^{h}
10	0	0.88±0.0062^{k}	0.30±0.0050^{c}	69.92±0.58^{d}
	5	1.12±0.0062^{g}	0.28±0.0086^{de}	65.35±0.61^{e}
	10	1.09±0.0040^{h}	0.23±0.0028^{f}	58.84±0.53^{g}
20	0	0.62±0.0086^{m}	0.39c0.0028^{a}	93.53±0.57^{a}
	5	0.93±0.0047^{j}	0.26±0.0040^{e}	69.40±0.56^{d}
	10	0.75±0.0047^{l}	0.28±0.0064^{dc}	77.35±0.62^{b}

Data are the means of four replicates (Mean ± SEM) and different letters indicate significant differences at $P < 0.05$ level.

The application of GA_3 diminished the detrimental effects of Cd on photosynthesis (Table 1). GA_3 is considered as a promoter for photosynthesis which through senescence retardation protects chloroplasts ultrastructure and chlorophylls. In addition, it was reported that GA_3 increased the activities of some enzymes of carbon metabolism such as Rubisco and enhanced transpiration rate and water use efficiency (Siddiqui *et al.* 2011).

Effects of Cd and GA_3 on RWC

The contents of RWC decreased along with increasing Cd concentrations in the nutrient solution (Fig. 1). Maximum decline in RWC contents were 21.18% and 39.02% in 5 and 20 µM $CdCl_2$, respectively. Whereas, with the addition of GA_3 increased RWC contents were shown in comparison to control plants.

Leaf RWC is described as a measuring factor for plant water content (Flower and Ludlow, 1986). It has been reported that Cd alters the plasma membrane permeability and disturbs the activity of plasma membrane aquaporins (Benavides

et al. 2005; Tamás *et al.* 2008). Gadallah (2000) suggested that the decrease in RWC contents could be due to inability of roots to restitute the water decline by transpiration through diminish of uptake surface.

On the other hand, the application of GA_3 in Cd-treated plants improved RWC contents (Fig. 1). This increase could be attributed to the role of GA_3 to enhance proline, glycinebetaine and other compatible solutes accumulations which have osmo-protective roles (Siddiqui *et al.* 2011).

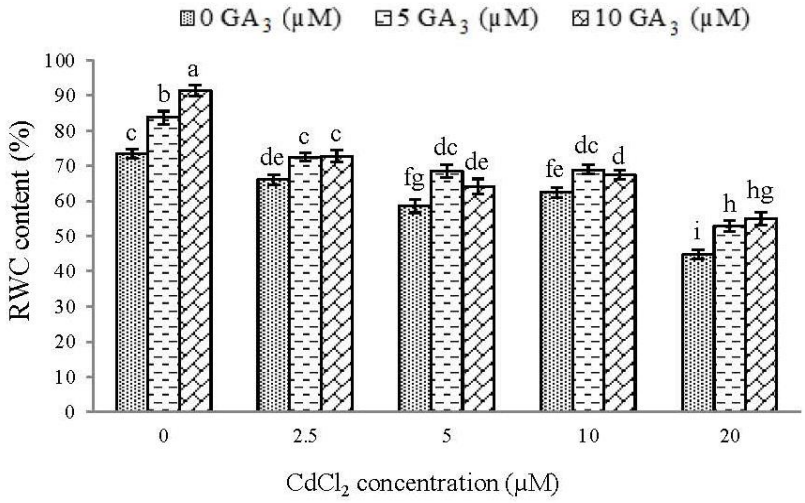

Figure 1. The effects of GA_3 application on the RWC content of Cd stressed plants. Data are the means of four replicates (Mean ± SEM) and different letters indicate significant differences at $P < 0.05$ level.

Effects of Cd and GA_3 on photosynthetic pigments

As results of Cd toxicity, decline in chlorophylls and carotenoids were observed (Table 2). Cd adversely impacts some enzymes involved in chlorophyll biosynthesis, such as protochlorophyllide reductase, ALA synthase and ALA dehydratase (Benavides *et al.* 2005; Siddiqui *et al.* 2011). The decrease in chlorophyll contents appears to be related to the antagonistic effects between uptake of Cd and other nutrients such as Fe and Mg which are involved in chlorophyll biosynthesis (Benavides *et al.* 2005). Interestingly, carotenoids act as non-enzymatic antioxidant components hence the decrease in carotenoids contents may be ascribed to the Cd-induced ROS production (Gill and Tuteja, 2010). The application of GA_3 increases the contents of chlorophylls and carotenoids (Table 2). These results agree with the findings of Siddiqui *et al.* (2011) and Saeidi-Sar *et al.* (2007), who reported that the content of chlorophylls was higher in plants exposed to GA_3.

Table 2

The effects of GA_3 application on the Chlorophyll *a*, Chlorophyll *b* and carotenoids contents of Cd stressed plants

$CdCl_2$ (μM)	GA_3 (μM)	Chlorophyll *a* (mg g^{-1} F.W.)	Chlorophyll *b* (mg g^{-1} F.W.)	Carotenoids (mg g^{-1} F.W.)
0	0	1.39±0.043^{d}	0.81±0.025^{c}	0.42±0.019^{d}
	5	1.73±0.053^{b}	0.93±0.027^{b}	0.53±0.023^{b}
	10	2.06±0.058^{a}	1.09±0.030^{a}	0.58±0.011^{a}
2.5	0	1.20±0.032^{f}	0.71±0.031^{de}	0.42±0.016^{d}
	5	1.52±0.043^{c}	0.80±0.012^{c}	0.51±0.016^{b}
	10	1.56±0.032^{c}	0.79±0.025^{c}	0.49±0.010^{cb}
5	0	0.95±0.046^{g}	0.59±0.020^{f}	0.34±0.019^{e}
	5	1.20±0.032^{f}	0.71±0.017^{de}	0.43±0.014^{d}
	10	1.32±0.025^{ed}	0.80±0.019^{c}	0.46±0.009cd
10	0	0.88±0.032^{g}	0.64±0.018^{f}	0.35±0.014^{e}
	5	1.14±0.030^{f}	0.74±0.017^{dc}	0.42±0.012^{d}
	10	1.26±0.026^{ef}	0.77±0.018^{dc}	0.46±0.013^{cd}
20	0	0.68±0.040^{h}	0.41±0.023^{g}	0.24±0.013^{f}
	5	0.90±0.026^{g}	0.65±0.012^{fe}	0.33±0.008^{e}
	10	0.88±0.025^{g}	0.59±0.014^{f}	0.35±0.013^{e}

Data are the means of four replicates (Mean ± SEM) and different letters indicate significant differences at $P < 0.05$ level.

Effects of Cd and GA_3 on total protein

Along with enhancement of Cd concentrations, total protein contents reduced (Fig. 2). Proteins are extremely sensitive to Cd-induced oxidative damage (Benavides *et al.* 2005). Decrease in proteins contents may be due to increase of the proteins denaturation involved processes in which enhancement of the protease activities is important (Wang *et al.* 2008). In addition, Cd alters the uptake and transport of Mn, K and N which are pivotal nutrient elements for protein biosynthesis (Benavides *et al.* 2005; Wang *et al.* 2008). Besides, Cd bonds to sulfhydryl groups in proteins, consequently, induces inhibition of enzymes activities and causes protein denaturation (Benavides *et al.* 2005).

On the other side, increase of the proteins contents in response to different GA_3 treatments was observed (Fig. 2). This increase may be due to the role of GA_3 in enhancement proline accumulation which acts as protein stabilizer and antioxidant molecule (Siddiqui *et al.* 2011). In addition, part of this increase could be attributed to the role of GA_3 in the increase of the biosynthesis of anti-stress protein such as phytochelatins and activities of antioxidant enzymes (Siddiqui *et al.* 2011).

Effects of Cd and GA_3 on proline content

The results showed the proline contents significantly promoted with increase of Cd concentrations (Fig. 2). Proline is an osmolyte and antioxidant molecule that has several roles in plant adaptation to stress conditions, such as detoxification of ROS and

stabilization of membranes and proteins (Wang *et al.* 2008; Siddiqui *et al.* 2011). It is known that free proline can bond to Cd leading to formation of non-toxic complexes Cd-proline (Siddiqui *et al.* 2011). In addition, Sumithra and Reddy (2004) suggested that the increase of proline contents could be due to increasing in the activities of Δ´-proline-5-carboxylate synthetase and Δ´-proline-5-carboxylate reductase enzymes.

On the other side, GA_3 treatments led to increase the proline contents (Fig. 2). This result conforms to findings of Khan *et al.* (2010) and Siddiqui *et al.* (2011), who reported higher proline accumulation in GA_3-treated plants.

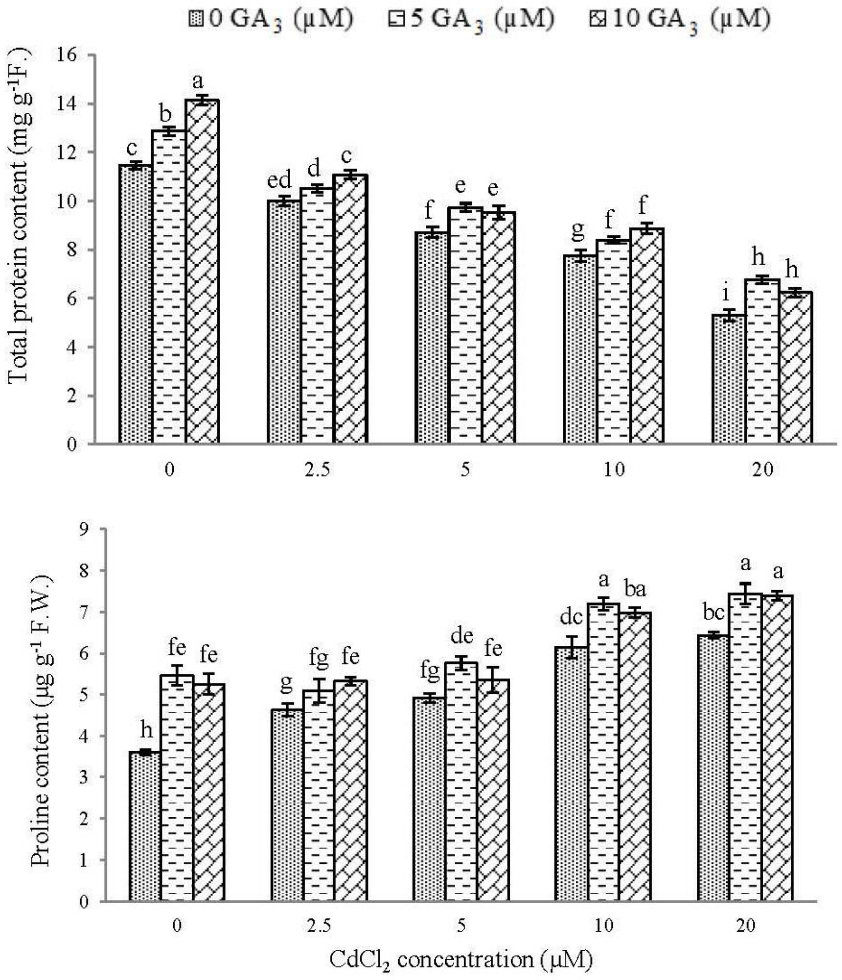

Figure 2. The effects of GA_3 application on the total protein and proline contents of Cd stressed plants. Data are the means of four replicates (Mean ± SEM) and different letters indicate significant differences at $P < 0.05$ level.

Effects of Cd and GA_3 on anthocyanin and total phenolic contents

With increasing Cd and GA_3 concentrations, increased anthocyanin and total phenolic contents were observed (Fig. 3). Anthocyanins are a group of pigments which are responsible for red, violet and blue colors in flowers, fruits and leaves of plants. It is known that anthocyanins elevation was induced against stress condition (Hale *et al.* 2001). Hale *et al.* (2001) reported that anthocyanins form metal-anthocyanin complexes sequestrated in the vacuoles of epidermal cells. In addition, anthocyanins have free radical scavenging roles and reduce the Cd-induced oxidative damage in plants (Hale *et al.* 2001).

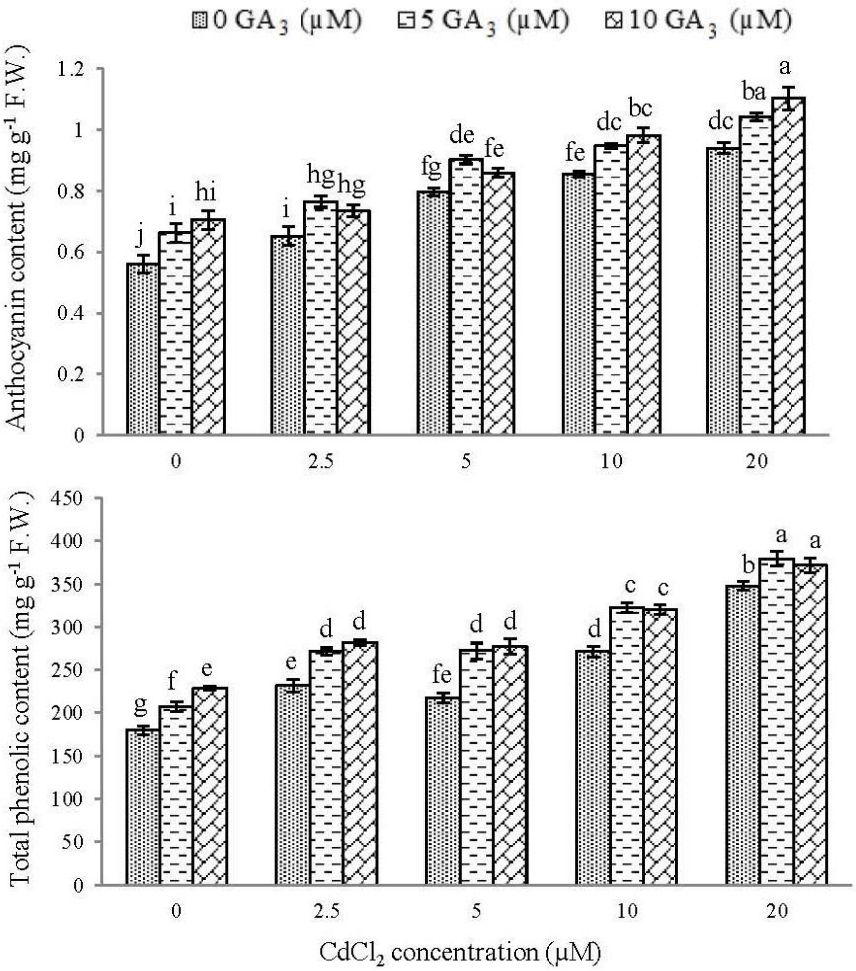

Figure 3. The effects of GA_3 application on the anthocyanin and total phenolic contents of Cd stressed plants. Data are the means of four replicates (Mean ± SEM) and different letters indicate significant differences at $P < 0.05$ level.

Phenolic compounds play an important role in plants adaptation to stress condition (Gill and Tuteja, 2010). They act as metal-chelators whose ability is the sequestration of toxic metals in the negatively charged sites of the cell wall (Macfie and Welbourn, 2000). In addition, increase of the phenolic compounds could be explained by the increased activity of phenylalanine ammonia-lyase under Cd toxicity. This enzyme has crucial roles in biosynthesis of phenolic compounds through producing the skeleton of cinnamic acid from phenylalanine (Kováčik *et al.* 2009).

Phenolic compounds have a pivotal role in detoxification of H_2O_2 and they are suitable substrates for peroxidases (Gill and Tuteja, 2010). Thus, increase of the phenolic contents in tomato plants exposed to GA_3 can be attributed to their roles in detoxification of ROS.

CONCLUSION

Based on the obtained results, it can be concluded that under Cd toxicity many of plant physiological activities such as photosynthesis are suppressed. However, GA_3 plays a fundamental role in the control of plant growth and development from germination to death. Therefore, its application helps plants tolerate Cd toxicity and ameliorate photosynthesis damage.

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ANALYSIS OF GENETIC DIVERSITY AND RELATIONSHIP AMONG NIGERIAN AND INDIAN ACCESSIONS OF *PENNISETUM GLAUCUM* AND *PENNISETUM PURPUREUM* BASED ON RAPD MARKERS

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In the present study, we investigated the genetic variations in pearl millet (*Pennisetum glaucum*) and napier grass (*Pennisetum purpureum*) accessions from Nigeria and India using random amplified polymorphic DNA (RAPD) markers. Genomic DNA extraction was carried out using QIAGEN DNeasy Kit and fragment amplification was performed by Polymerase Chain Reaction. 60.63% of the amplified loci by 20 markers were polymorphic while 39.37% were monomorphic and the percentage polymorphism per primer ranged from 33.33–72.72%. The biplot analysis showed that the markers effectively separated the accessions into groups based on genetic similarity. Cluster analysis classified the accessions into two broad groups: a group which comprised all napier grass accessions and the other the pearl millet. The pearl millet group had sub-clusters which are mixtures of Nigerian and Indian accessions and were linked to the napier grass accessions. TAYABI and JALGONE which were sourced from farmer's field were genetically similar and distinguished from other pearl millet accessions. The study revealed intra and inter-specific variations among the accessions and the occurrence of Nigerian and Indian accessions in a cluster indicated they are genetically related and possibly from the same progenitor but could have be separated by a geographical or ecological isolation mechanism.

Keywords: DNA polymorphism, molecular genotyping, interspecific variability, napier grass, RAPD.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L. R. Br.) and napier grass (*Pennisetum purpureum* Schum.) are the two economically most important members of the genus *Pennisetum* (Poaceae) which comprises about 140 species widely distributed around the world (Bodgan, 1977; Chaudhary, 1989). Napier grass is an important forage crop in the tropical and subtropical regions. The grass is valued for its high biomass yield, forage quality, pest resistance and perennial nature (Farrell *et al.*, 2002). Napier grass is evasive and grows rapidly, its enormous degradable biomass yield

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makes it a potential candidate for bioenergy conversion (Azevedo *et al.*, 2012; Dowling *et al.*, 2013). Pearl millet on the other hand is an annual grass grown mainly for grain but also utilized as forage. It is a major warm season cereal, highly drought tolerant and ranked fifth most important food-grain crop following rice, wheat, maize and sorghum. Pearl millet is grown on more than 26 million hectares (ha) in the arid and semi-arid tropical regions of Asia and Africa where it serves as staple food (FAO and ICRISAT, 1996; Rai *et al.*, 2009). In the USA, Australia and parts of South America, pearl millet is grown mainly as forage crop. However, there is increase interest in growing the pearl millet as grain crop to supplement utilization of "super cereals" as feed for poultry and livestock (Hanna *et al.*, 2004).

In the past decades, there have been initiatives for pearl millet and napier grass improvement using a classical plant breeding approach for different traits. The prerequisite for attaining this goal involves screening of different germplasms for desired trait by using morphological, biochemical and anatomical features. These features can be influenced by environmental factors, farming practices, and often they require much longer time of study. Hybridization of pearl millet and napier grass could produce hybrids of combined characters for the purpose of grain, forage and biomass yield to meet the demands for food security and clean energy through biomass conversion to biofuels (Hanna *et al.*, 2004; Dowling *et al.*, 2014).

Consequently, there is need to characterize accessions of these species with a view to identify traits of agronomic importance whose variability will be useful for breeding purposes to create the genetic framework for understanding variability that is not influenced by the environment in the study of the plant diversity. Utilisation of various molecular markers to assess the genetic variability in plants has proved to be precise, veritable and effective in distinguishing and revealing inter and intra-specific variations and relationships in plants (Wendel *et al.*, 1992; Azevedo *et al.*, 2012). Molecular characterization and diversity study using DNA polymorphism have been increasingly employed to characterize, identify and clone novel germplasms for breeding programmes (O'Neill *et al.*, 2003). Several DNA marker systems have been used for these purposes. PCR-based molecular markers such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are commonly used among others because they save time (Colombo *et al.*, 1998; Zhang *et al.*, 2005; Babu *et al.*, 2007) and their submission does not need prior sequence information (Rana *et al.*, 2013).

An extensive RAPD study conducted by Williams *et al.* (1990) provided a considerable number of markers which can be used for various purposes like cultivar analysis and species identification in plants. Also, AFLP markers have been widely used to identify various accessions, landraces, population diversity and relatedness. Comparisons of different DNA markers for diversity studies in *Zea mays* (Garcia *et al.*, 2004), finger millet (Panwar *et al.*, 2010), wheat (Chao *et al.*, 2007) etc. have been carried out to evaluate the relative efficiencies of the different techniques available. In addition, DNA fingerprinting studies to assess genetic purity with RAPD have already been conducted in cotton (Soregaon *et al.*, 2004)

and cross species amplification between pearl millet and napier grass using SSR markers was reported by Azevedo *et al.* (2012).

Furthermore, accessions of pearl millet, napier grass and their interspecific hybrids (*Pennisetum purpureum* × *Pennisetum glaucum*) have been characterized by morphological, chemical, cytogenetic and molecular traits (Techio *et al.*, 2006; Davide *et al.*, 2007; Pereira *et al.*, 2008). Nonetheless, there is still wide gap in the study of variation and diversity among the two species. Details of their genetic variation through morphological and molecular characterization have potential for elucidating the phylogenetic relationship of the species and their inter and intra-specific variations. In view of these, we assessed the genetic diversity of accessions of pearl millet and napier grass from Nigeria and India using RAPD markers. The aim of this study is therefore to provide baseline information on inter and intra-specific variation that would be useful as genetic resources for breeding and evolutionary studies of napier grass and pearl millet.

MATERIAL AND METHODS

Plant materials

A total of twenty-nine accessions consisting of twenty-four pearl millet and five napier grass were used for this study (Table 1). The pearl millet were collected from International Crops Research Institute for the Semi-Arid Tropics, (ICRISAT), Patanchera, Andhra Pradesh, India; National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria and farmer's field in Gujarat State of India. Clones (stem cuttings) of two napier grass genotypes were collected from the wild population in Omuo-Ekiti, Ekiti state of Nigeria and three others were obtained from the Department of Forage Crop, Tamil Nadu University of Agriculture, Coimbatore, India.

DNA Extraction

Fresh young leaves of each accession grown in the green house at C. G. Bhakta Institute of Biotechnology. Bardoli, India were used for genomic DNA isolation. About 300 mg of fresh leaf tissue was grounded into a fine powder in prechilled mortar using liquid nitrogen and genomic DNA from individual accession were extracted using DNeasy Plant Mini Kit (QIAGEN, USA). The DNA extraction was performed in accordance with the manufacturer's instructions. The precipitated DNA was dissolved in 100 µl of elution buffer. The quality and quantity of the DNA were checked using a spectrophotometer and agarose gel (0.8%) electrophoresis, respectively. The absorbance ratio of DNA sample between 260 and 280 nm was recorded and the quality of the genomic DNA was confirmed. The purified DNA samples were stored at 4ºC till use.

PCR amplification

RAPD marker amplification was performed as described by (Sharma *et al.*, 208) using 23 decamer random primers. The PCR reaction was carried out with 25 µL volume containing 12.5 µL reaction mixture (1 x reaction buffer with 2.0 mM $MgCl_2$, 200 µM each of deoxynucleotides (dNTPs), 0.5 µL of *Taq* polymerase), 1 µL of 10 pMole primer 1 µL of 50 ng genomic DNA. The total reaction mixture volume was made up to 25 µL with nuclease free water. The reaction tubes were placed in a Thermocycler (Eppendorf, USA). The PCR protocol was run with a cycle of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 sec, annealing at 27°C (annealing temperature of each decamer) for 50 sec and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min.

The amplified products were resolved by electrophoresis (1.5 % agarose gel) and the amplified loci were visualized under UV light and imaged on a Gel Documentation System (Bio-Rad, US). The procedure was performed with three replicates for each of the sample and selected primer. Fragment sizes of the amplification products obtained from the RAPD markers were determined from the gel by comparison with standard molecular weight marker ladder-low range 100 basepair DNA Ruler (Thermoscientific, USA).

Data analysis

The gel image of the amplified PCR products were scored across the lanes comparing their respective molecular weights with the gene ruler. Intense and clear bands were scored for data matrix as 1 for present or 0 for absent. Only well-defined and consistently reproducible bands in three independent amplifications were included in the final analysis. Module analysis was performed with NTSYS-PC and Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) using MEGA 4.0 software while genetic differentiation and Shannon's index (I) were determined using POPGENE 3.2.

RESULTS

The RAPD diversity study of the 29 accessions of pear millet and napier grass showed high degree of polymorphism. The code, sequences and other properties of the markers used are presented in Table 2. Twenty markers produced distinct and reproducible bands among the markers tested and the amplified PCR product showed arrays of monomorphic and polymorphic bands. Amplification profiles of the accessions with 20 random primers produced a total of 178 loci consisting of 2140 alleles with average of 9.01 alleles per loci with amplicon size ranging from 150 to 2,500 base pair (bp). The number of DNA fragments or bands produced per accessions

by the primers ranged from 1 to 13 (Table 3). A single band was amplified by primer OPH-20 on accession IP12556, however, a minimum of four (4) bands were amplified from some accessions by primers OPH-19, ADG-4, RAPD-10, RAPD-036, RAPD-030, RAPD-014 and OPD-6 while other primers amplified at least five bands. Although, primer RAPD-09 recorded minimum of 4 bands amplification on some accessions, it also produced 13 bands on some other accessions. Primers RAPD-04 and RAPD-09 amplified the highest number of bands.

Out of the 2140 total alleles generated by the 20 primers, 1297 (60.63%) were polymorphic while 843 (39.37%) were monomorphic and the percentage polymorphism per primer ranged from 33.33 – 72.72% (Table 3). The highest percentage polymorphism (72.72%) was obtained with primer LC-71 as against primers RAPD-12 and RAPD-036 which had least (33.33 %) percentage polymorphism each. With the exception of primers RAPD-12 and RAPD-036, all primers produced polymorphic bands of 50% above.

The Dominance indices ranged from 0.03 to 1.00 for the markers used. The marker LC-71 recorded the highest Dominance index of 1.00 in contrast to RAPD-04 were RAPD-036 which had dominance values of 0.03 each. Also, low dominance index factors of 0.04 were obtained with markers RAPD 09, RAPD-04 and OPD-6. Shannon index (H) analysis revealed values that ranged from 1.31 to 3.38. Marker ADG-4 had the highest value of 3.38 while the least values of 1.31 were obtained with primer OPB-1. Out of the markers tested, seven primers; viz: OPH-20, ADG-4, OPK-4, OPB-1, LC-71, RAPD- 010 and RAPD-011 had Shannon index less than 2.00 while the rest of the tested primers recorded Shannon index above 2.00 as shown in Table 3.

The Biplot analysis of the markers on the accessions revealed distribution of the accessions in four quadrants (Figure 1). The first quadrant consisting of napier grass accessions COM-CO3 and COM-CO2, the distributions of these two accessions in a quadrant were marked by OPH-19, RAPD-030, RAPD-036 and RAPD-011 primers. In contrast, the second quadrant hada mixture of pearl millet and napier grass accessions. Napier grass accession Omuo-Green overlapped COM-CO4 and closely located on the same plain with Omuo-purple but on the far end of the second quadrants. Meanwhile, pearl millet accessions NGB00537, NGB00528 and IP22269 were located close to the centroid (Figure 1). The accessions placed in quadrant-II were delimited by markers OPB-1 and RAPD-2. Quadrant-III comprised of ten pearl millet which are a mixture of Nigerian and Indian accessions and 8 markers were indicated in the quadrant's distribution. Similarly, quadrant-IV hadan (eleven) mixture of India and Nigerian pearl millet accessions marked by 6 RAPD markers. Two wild accessions (JALGONE and TAYABI) of India origin were located at far distance axis of the quadrant.

Cluster analysis generated from matrix similarity analysis of the 29 accessions of pearl millet and napier grass based on 20 RAPD markers revealed two major groups (Figure 2). Group 1 consisted of all the napier grass accessions

and it is sub-divided into two clusters (1a and 1b). Cluster 1a had the two accessions (Omuo-Green and Omuo-Purple) from Nigeria while cluster 1b comprised napier grass accessions from India (COM-CO2, COM-CO3 and COM-CO4). However, the Indian napier grass accessions further segregated with COM-CO2 on a sole divide while others (COM-CO3 and COM-CO3) remained similar.

On the other hand, the second group (Group 2) sub-divided into 2a and 2b (Figure 2). The 2a group split into 2 sub-clusters (Fig. 2) with a sub-cluster comprising four pearl millet accessions of which two (BAJARA and BALKUVE) are from the farmers' field while the other two (IP3495 and IP3616) are ICRISAT accessions. The second sub-cluster also separated into two: one of the minor clusters had five members which are a mixture of Nigerian and Indian accession, the second one consisted of two micro-clusters with three and four accessions respectively.

DISCUSSION

The RAPD marker employed in diversity studies of napier grass and pearl millet in the present study showed sequence related amplified polymorphism providing information on diversity of the accessions. High polymorphism obtained in the study conformed to earlier reports of molecular markers characterizations of napier grass and pearl millet (Daher *et al.*, 2002, Xie and Lu, 2005; Pereira *et al.*, 2008; Harris *et al.,* 2009). However, data comparison between markers for allelic and loci informativeness is difficult due to different markers used. For instance, some markers had fragment amplified in all the accessions while some only amplified in a number of accessions. Primer RAPD-09 which had four bands with an accession produced thirteen bands with other accession thus reduces the uniformity in allelic frequency distributions of the markers. A similar result was reported on cross amplification of microsatellite marker on napier grass by Azevedo *et al.* (2012)**.** The discrepancies in markers amplification could be due to pipetting error, during electrophoresis, or low profile of the affected markers which was also reported by Lowe *et al. (*2003).

The polymorphism range of 44.44 - 72.72 exhibited by the markers in this study, though slightly less than those reported by Govindaraj *et al.* (2009) which showed that the tested markers are effective and informative in providing relationship trends among the accessions considered. This further corroborated earlier report of high polymorphic band amplification by RAPD in napier grass genotyping and concluded that RAPD was an effective marker for diversity study in the crop (Russell *et al.*, 1997). In an earlier study, Lowe *et al.*, (2003) compared hybrid napier grass and farm clone genotypes and distinguished them with RAPD primers which produced high polymorphism. Also, high polymorphism has been reported in molecular characterization of finger millet (Gupta, 2010), okra (Nwangburuku *et al.*, 2011; Haq *et al.*, 2013), pepper (Aslam *et al.*, 2014) and

wheat (Naghavil *et al.*, 2004). In all the cases mentioned above, polymorphism of the amplified loci was the basis for genetic diversity which also applied in this study. Although studies on agro-morphological and cytological traits have been conducted to determine genetic similarity between pearl millet and napier grass (Morakinyo and Adebola, 1991; Barbosa *et al.*, 2003, Hash *et al.*, 2003; Techio *et al.*, 2006). However, less information is available on molecular marker diversity of the species. The present study aligned with the findings of Azevedo *et al.* (2012) and Xie *et al.* (2009) who had earlier investigated genetic variability of napier grass and its closely related cultivars by microsatellite cross amplification and SRAP respectively. The results obtained could allow a deeper exploitation of the molecular information provided by the markers.

The distribution of accessions in different clusters and co-ordinates by biplot analysis showed the involvement of markers in separating accessions in quadrants. The spatial closeness of the accessions indicated their genetic similarity and the dispersion of the markers from the centroid reflects their effectiveness in delimiting the accessions. Co-occurrence of Indian and Nigerian accessions in the same plane suggested a common ancestor and further led credence to West African origin of pearl millet (Jauhar, 1981; Barbosa *et al.*, 2003). The observed diversity could have arisen from geographical isolation and selected mutations. The overlapping of OMUO-GREEN and COM-CO4 which are related to OMUO-PUPLE indicated the accessions are genetically similar. Nwangburuka *et al.* (2011) and Dowling *et al.* (2013) in their independent studies demonstrated genetic similarity in genotypes of napier grass and pearl millet. However, distant location of JAGONE, BALKUVE, and TAYABI from other pearl millet accessions implies existence of genetic distance among the accessions as previously observed in some accessions of pearl millet (Lowe *et al.*, 2003). This may be due to accumulation of some genes through selection resulting from domestication of the accessions by local farmers. Meanwhile, the obtained marker efficiency as revealed by biplot analysis supported RAPD markers as useful tool for the initial assessment of intraspecific genetic variation (Virk *et al.*, 1995).

Cluster analysis by dendrogram separated the accessions into groups which further partitioned into nine clusters. Clustering of all napier grass together irrespective of their sources showed their remarkable genetic similarity. The mixture of Nigerian and Indian pearl millet accessions in a cluster further reinforced the postulate of a common progenitor for the accessions (Jauhar and Hanna, 1998). Interestingly, JALGONE and TAYABI (farmer' field accessions) congregated into a micro sub-cluster which showed they are genetically related but separated from other Indian accessions and this leads to gradual development of ecotypes based on farmers preference as suggested above. The clustering pattern obtained in this study was in consonance with clusters produced by SRAP markers reported by Xie *et al.* (2009) and a similar one produced from SSR markers analysis (Sumathi *et al.*, 2013).

CONCLUSION

Accurate assessment of genetic diversity is important in crop breeding and improvement. Adequate genetic information would identify potential parental combinations to create segregating progenies with maximum genetic variability for further selection for improvement. The present work carried out genetic diversity of analysis *Pennisetum glaucum* (pearl millet) and *P. purpureum* (napier grass) showed that inter and intra- molecular diversity exists in the accessions of pearl millet and napier grass accessions studied. Clustering of pearl millet from Nigeria with India accessions confirms that they are genetically related and possibly from same progenitor but could be separated by a geographical or ecological isolation mechanism. Analysis of genetic diversity revealed association between pear millet and napier grass which supports that the latter belongs to the secondary gene pool of the former and that they could integrate their genetic materials.

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Table 1

Common name, source and nature of genotypes of pearl millet and napier grass used for genomic size and ploidy analysis

SN	Genotype	Common name	Source of collection
1	IP3616	Pearl millet	ICRISAT
2	IP3495	Pearl millet	"
3	NGB00463	Pearl millet	NACGRAB
4	BALKUVE	Pearl millet	Gujarat, India *
5	BAJARA	Pearl millet	Gujarat, India*
6	NGB00616	Pearl millet	NACGRAB
7	IP4133	Pearl millet	ICRISAT
8	NGB01263	Pearl millet	NACGRAB
9	IP22281	Pearl millet	ICRISAT
10	IP12556	Pearl millet	"
11	NGB00458	Pearl millet	NACGRAB
12	NGB00531	Pearl millet	"
13	IP22271	Pearl millet	ICRISAT
14	NGB00476	Pearl millet	NACGRAB
15	IP22268	Pearl millet	ICRISAT
16	TAYABI	Pearl millet	Gujarat, India*
17	JALGONE	Pearl millet	"
18	NGB00551	Pearl millet	NACGRAB
19	IP17862	Pearl millet	ICRISAT
20	IP3122	Pearl millet	"
21	NGB00528	Pearl millet	NACGRAB
22	IP22269	Pearl millet	ICRISAT
23	NGB00528	Pearl millet	NACGRAB
24	NGB00537	Pearl millet	"
25	OMUO-GREEN	Napier grass	Ekiti-State, Nigeria**
26	OMUO-PURPLE	Napier grass	"
27	COMBUS-CO2	Napier grass	Tamil-Nadu, India***
28	COMBUS-CO3	Napier grass	"
29	COMBUS-CO4	Napier grass	"

NACGRAB: National Centre for Genetic Resources and Biotechnology, Ibadan, Nigeria
ICRISAT: International Crops Research Institute for the Semi-Arid Tropics, Patanchera, Andhra Pradesh, India
* Collected from farmers' field, in Surat District of Gujarat State, India.
* *Omuo-Ekiti, Ekiti State, Nigeria
*** Department of Forage Crop, Tamil Nadu University of Agriculture, Coimbatore, India

Table 2

Primer code, name, sequence, dilution factors and other properties of the 20 RAPD markers used for diversity and molecular characterization of twenty-nine accessions of napier grass and pearl millet

S/N	Code	Seq Name	Primer Seq 5'- 3'	Tm (°C)	% GC
1	BA00241966	OPD-8	GTGTGCCCCA	27	70
2	BA00241967	OPH-19	CTGACCAGCC	27	70
3	BA00241968	OPK-19	CACAGGCGGA	27	70
4	BA00241969	OPH-20	GGGAGACATC	25	60
5	BA00241970	ADG-4	CCCGCCGTTG	29	80
6	BA00241971	OPK-4	CCGCCCAAAC	27	70
7	BA00241972	OPB-1	GTTTCGCTCC	25	60
8	BA00241973	LC-71	TGCCGAGCTG	27	70
9	BA00241974	RAPD-010	CCACACTACA	23	50
10	BA00241975	RAPD-012	CGGCCACTGT	27	70
11	BA00241976	RAPD-015	CGGCCCCGGC	33	100
12	BA00241977	RAPD-04	CGGAGAGCGA	27	70
13	BA00241978	RAPD-09	GACGGAGCAG	27	70
14	BA00241979	RAPD-036	GAAGAACCGC	25	60
15	BA00241980	RAPD-021	GACGGATCAG	25	60
16	BA00241981	RAPD-011	CGGAGAGCCC	29	80
17	BA00241982	RAPD-031	GGGTAACGCC	27	70
18	BA00241983	RAPD-030	GGACTGGAGT	25	60
19	BA00241984	RAPD-014	CGGCCCCGGT	31	90
20	A00241965	OPD-6	GGGAATTCGG	27	60

Tm = Melting temperature; %GC = percentage Guanine-Cytosine

Table 3

Band amplification, polymorphism and molecular diversity index profile of the 20 RAPD markers used for diversity study of twenty-nine accessions of pearl millet and napier grass

S/N	Marker ID	Sequence (5'-3')	Band amplification		Polymorphism		Dominance factor	Shannon index (H)
			Min	Max	Bands	%		
1	OPD-8	GTGTGCCCCA	5.00	8.00	5.00	62.50	0.17	3.17
2	OPH-19	CTGACCAGCC	4.00	7.00	5.00	71.42	0.13	2.09
3	OPK-19	CACAGGCGGA	5.00	9.00	6.00	66.67	0.10	2.30
4	OPH-20	GGGAGACATC	1.00	11.00	6.00	54.54	0.20	1.61
5	ADG-4	CCCGCCGTTG	4.00	8.00	5.00	62.50	0.25	1.38
6	OPK-4	CCGCCCAAAC	6.00	9.00	6.00	66.67	0.25	1.45
7	OPB-1	GTTTCGCTCC	5.00	9.00	5.00	55.56	0.24	1.31
8	LC-71	TGCCGAGCTG	6.00	11.00	8.00	72.72	1.00	1.38
9	RAPD-010	CCACACTACA	4.00	8.00	5.00	62.50	0.20	1.60
10	RAPD-12	CGGCCACTGT	6.00	9.00	4.00	44.44	0.56	2.31
11	RAPD-15	CGGCCCCGGC	5.00	10.00	6.00	60.00	0.05	2.43
12	RAPD-04	CGGAGAGCGA	5.00	13.00	8.00	61.54	0.03	3.36
13	RAPD-09	GACGGAGCAG	4.00	13.00	9.00	69.23	0.04	3.36
14	RAPD-036	GAAGAACCGC	4.00	10.00	3.00	33.33	0.03	2.17
15	RAPD-021	GACGGATCAG	5.00	9.00	5.00	55.56	0.11	2.19
16	RAPD-011	CGGAGAGCCC	6.00	10.00	6.00	60.00	0.11	1.94
17	RAPD-031	GGGTAACGCC	5.00	8.00	4.00	50.00	0.14	2.56
18	RAPD-030	GGACTGGAGT	4.00	6.00	4.00	66.67	0.08	3.17
19	RAPD-014	CGGCCCCGGT	4.00	10.00	7.00	70.00	0.04	3.18
20	OPD-6	GGGAATTCGG	4.00	9.00	5.00	55.56	0.04	3.21

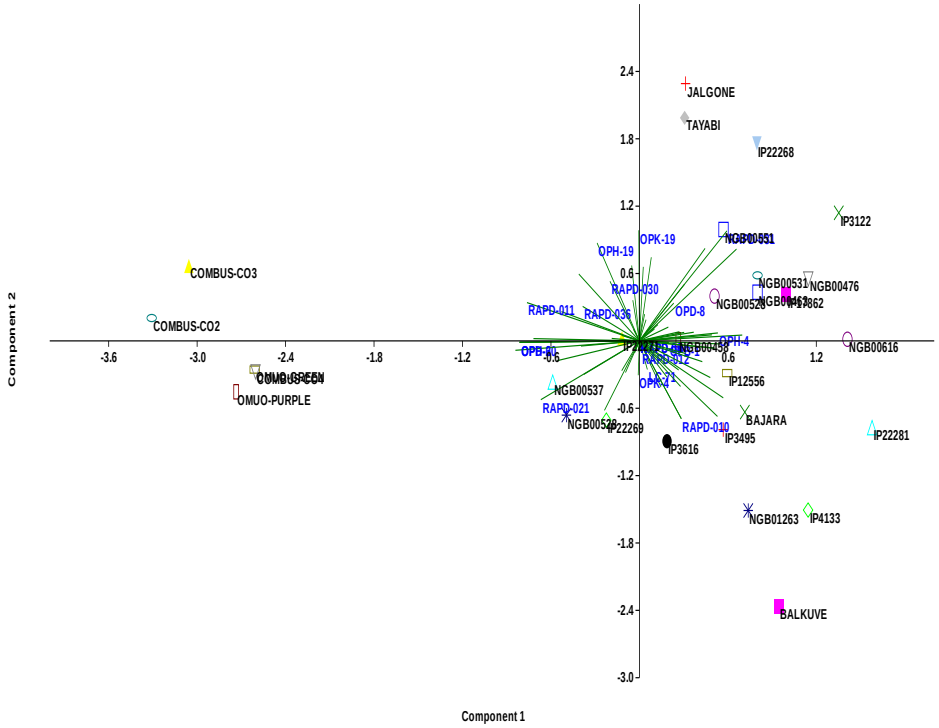

Figure 1. Bootstrapped Biplot of twenty-nine accessions of pearl millet and napier grass characterized by twenty RAPD primers for diversity and genotyping analysis (at $p<0.05$).

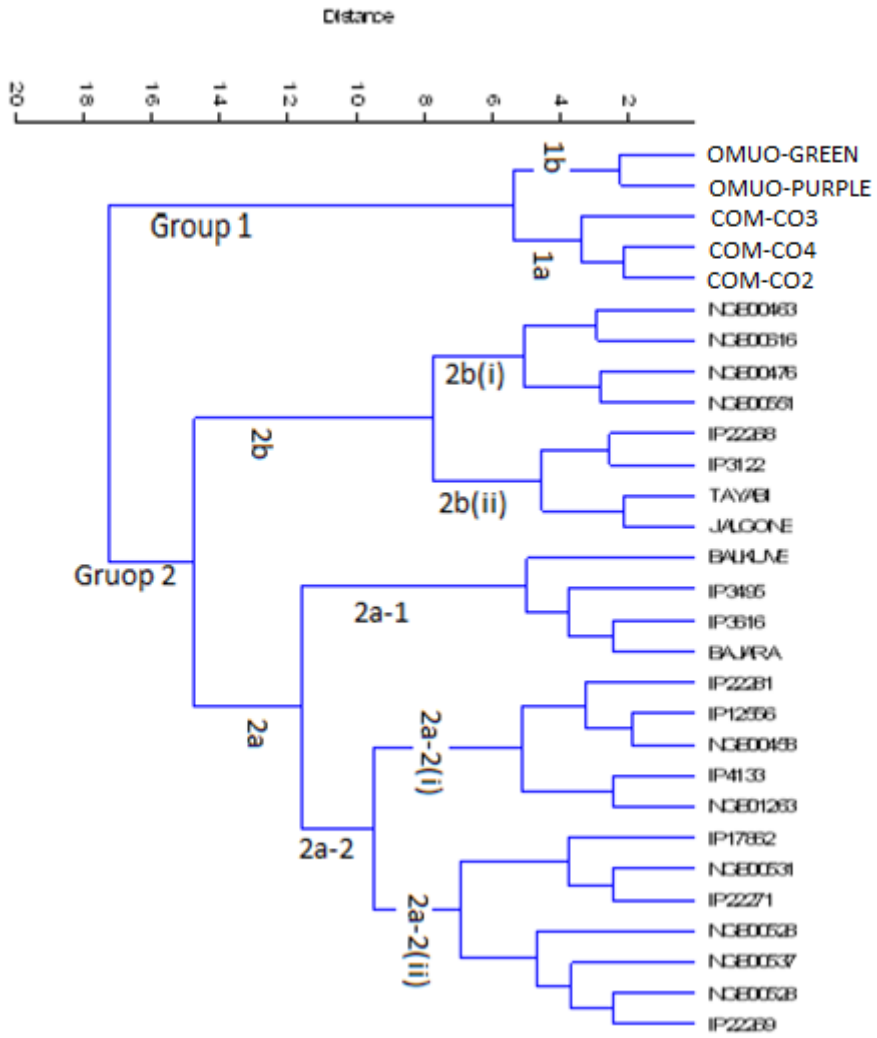

Figure 2. Cluster diagram and genetic grouping of twenty-nine accessions of napier grass and pearl millet based on amplified loci by twenty RAPD markers.

Halophiles: *Biodiversity and Sustainoble Exploitation*, in: *Sustainable Development and Biodiversity* **6.** Springer International Publishing, Switzerland, 2015, D.K. Maheshwari, M. Saraf (eds.), ISBN- 978-3-319-14595-2-5, 456 p.

"Halophiles: Biodiversity and Sustainable Exploitation" belongs to the Series Sustainable Development and Biodiversity, volume 6. The book edited by Dinesh K. Maheshwari and Meenu Saraf contains, apart from an introduction, 16 chapters written by well-known scientists from several countries (Algeria, Egypt, Germany, India, Iran, Italy, Kingdom of Saudi Arabia, Japan , Kuweit, Mexico, Romania, Turkey and the USA) and a useful index. One group of chapters is focused on Actinobacteria with emphasis on their biodiversity, (including quorum sensing system), antimicrobial and biocatalytic potential, as well as on their biotechnological exploitation (production of different types of enzymes, hydrocarbon degradation, heavy metal bioremediation and biominig, reduction of azo-dyes, etc.) and application of halophilic and halotolerant Actinobacteria in agriculture. Another group of chapters deals with their enzymes: hydrolytic enzymes in halophilic bacteria, properties and biotechnological potential; isolation and screening of halophilic bacteria for production of hydrolytic enzymes; perspectives and application of halophilic enzymes as well as extracellular proteases from halophilic and haloalkaliphilic bacteria: occurrence and biochemical properties. Special attention is devoted to the relationship between halophiles and nanotechnology, the two chapters presenting biosynthesis of nanoparticles from halophiles and the biomolecules of halophilic microorganisms in the frame of bio(nano) technologies. Two chapters concern beneficial usages of halophilic microorganisms and their potentials and applications in biotechnology, whereas other three chapters are focused on precise applications, such as: bioinoculants for sustainable agriculture in coastal saline soil, the restoration of plant growth under salt stress or microbial hydrocarbon-removal under halostress. Each chapter is written in a very clear style and has reached also very useful references; there are 43 black and white illustrations and 11 in color. Sometimes, there are few overlappings in the content of some chapters, emphasizing the professional personalities of contributors. In my opinion the book "Halophiles: Biodiversity and Sustainable Exploitation" is a very valuable reading for students as well as for already known scientists working in the field.

Ioan I. Ardelean

Microbial Factories: Volume 1 - *Biofuels, Waste treatment*. 2015, Kalia V.C. (Ed), Springer, India, ISBN 978-91-322-2597-3, 353p.

The book "Microbial Factories: Volume 1 – Biofuels, Waste treatment" contains 19 very interesting chapters. After two introductory chapters on some aspects of the state-of-the art in this domain, one main topic of this book concerns treating municipal wastes with generation of valuable chemicals and/or energy covered by four chapters, the production of molecular hydrogen is treated in three chapters and chemical biosynthesis in other three chapters (1, 3-, propanediol; cellulose, polyhydroxyalkanoate**)**. Other topics concern bio-methane in two chapters, electric energy production with biofuel cells (two chapters) and different important aspects on biodiesel production (enzymatic trans-esterification, residual glycerol reuse), and bioremediation of different pollutants (pesticides, arsenic). The book is written by reputed scientists in their field; however, the illustration of this book comprises only 13 black and white illustrations and 37 in color. I hope that the volumes to come will offer to the reader – the editor stresses on the fact that the book is dedicated to the so-called *Ignited Minds* and I fully agree with him – not only a dense and informative text, but also more useful and helpful illustrations. In my opinion, the book "Microbial Factories: Biofuels, Waste treatment" is not only an excellent scientific lecture, but also a working instrument to be found on the laboratory bench of scientists and on the *bureau* of responsible policymakers active in wastewater treatment and biofuels production.

Ioan I. Ardelean