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Review

Cryoconservation of plant germplasm native to Brazil

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The preservation of biological material at -196°C, that is, at liquid nitrogen temperature, or its vapor (between -150 and -178°C), is a long-term storage procedure called cryopreservation or cryoconservation. This article reports studies made in Brazil for cryoconservation of native plant species and highlights what might be the gap in this area of germplasm conservation. Most studies use seeds as plant material, and the great majority of those is orthodox, which means that countless species are being left out of this conservation effort, such as those with recalcitrant seeds and plants that do not produce seeds. Many articles addressed in this present work studied species threatened by anthropogenic activities and chose cryoconservation as a way to safeguard their germplasm. The Brazilian cryobanks, on the other hand, are still developing, with many studies yet to be made and many accessions yet to be incorporated to collections in order for them to achieve an ideal stage of operation.

Key words: Biodiversity, biotechnology, conservation, cryopreservation, preservation, seed.

INTRODUCTION

Plant genetic resources consist in a natural reserve of genes with potential use for sustainable produce such as food, fibers and medications, is essential to mankind. Genetic erosion is characterized by gradual loss of this genetic reserve, emerging from disorganized growth of human population and uncontrolled exploitation of the ecosystems and their natural resources, and also from the evolutionary process species undergo in nature through natural selection and genetic drift. Such loss can be mitigated through long-term conservation techniques with as much as possible genetic and biological integrity (Santos, 2001; Raven and Havens, 2014).

Thus, *in situ* and *ex situ* conservation of germplasm from domestic crops and wild relatives of agronomic species, as well as native species with still unexplored potentials, is proposed in sight of the destruction of biodiversity which has been observed nowadays and the climate changes planet Earth is going through. Con-

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servation of plant genetic diversity is imperative in face of such threats and will enable mankind to reestablish species in the wild when conditions are favorable (Raven and Havens, 2014). Furthermore, the conservation of biodiversity is imperative for both long known species as well as recently discovered ones, since the genetic resources of a species may not be of interest to mankind at present but become fundamental for solving problems that already exist and the ones that may arise in the future (Gonzaga et al., 2003).

Ex situ conservation, that is, the conservation that takes place outside of the natural environment in which the species occur, allows for medium to long term preservation of the stored species genetic variability and their anatomic, physiological and biochemical study to support strategies of reintroduction, genetic engineering and education (Heywood and Iriondo, 2003) that is accomplished by preserving a representative sample of the natural populations in germplasm banks, like, for instance, *in vitro* banks, seed banks, cryobanks, or field collections. Seeds, pollen and spores of plants are adequate for medium to long term conservation because of their natural adaptation to sustaining viability for extended periods of time (Heywood and Iriondo, 2003).

In situ conservation, however, being the conservation of species in the environment where they naturally occur, preserves physical and biological processes which *ex situ* conservation is incapable of safeguarding, such as evolution and ecological relationships, which, in turn, makes them complementary and essential strategies (Maxted et al., 1997; Raven and Havens, 2014). Therefore, *in situ* conservation depends on the maintenance of natural populations and their careful monitoring, because just creating conservation areas does not guarantee the survival of species, particularly threatened species (Heywood and Iriondo, 2003).

Since *in situ* conservation of endemic species is based in theoretical actions that are not strictly followed, applied or respected, complementary strategies like the creation of *ex situ* germplasm banks are recommended (Tarré et al*.,* 2007). The preservation of biological material at -196°C, that is, at liquid nitrogen temperature, or its vapor (between -150 and -178°C), is a long-term storage procedure called cryopreservation or cryoconservation. It is based on the principle that under ultralow temperatures of -196°C all metabolical processes are essentially paralyzed and kept in a latent state, consequently providing indefinite preservation (Medeiros and Cavallari, 1992).

Cryoconservation can be applied to a series of plant materials such as suspension cultures, calli, shoots, somatic embryos or embryonic axes, fern gametophytes, axillary buds and seeds (Sarasan et al., 2006). Among these, the storage of seeds is the most used method of *ex situ* conservation, because seeds are the natural structures of plant reproduction and each one can represent a genetically distinct organism (Tarré et al*.,* 2007). Seeds may be stored for indefinite amounts of time in conditions which reduce their metabolism and deterioration (Tarré et al*.,* 2007; Hartmann et al., 2011) as well as prolong their long-term viability (Salomão, 2002; Wetzel et al. 2003; Lima et al., 2008), besides, depending on the species, being small in size and occupying little space.

The evaluation of seed behavior after storage is the first step for the establishment of ideal storage conditions, as well as desiccation tolerance and freezing (Tarré et al*.,* 2007). There may be abnormal germination or death from internal injuries, which may be related to certain seed traits such as size, water content and chemical com-position (Cromarty et al., 1982; Santos, 2001; Goldfarb et al., 2010; Silva et al., 2011). Thus, the effect of freezing in liquid nitrogen should be analyzed for each and every species targeted for a cryoconservation study.

Seed cryoconservation is advantageous for long-term conservation of tropical and subtropical forest biodiversity because the stages of embryo isolation and *in vitro* manipulation are not necessary. Even for orthodox and intermediary seeds, whose conservation is less problematic then for recalcitrant seeds, cryoconservation preserves seed longevity as long as liquid nitrogen levels are maintained, whereas dry storage of seeds at −20°C in banks for long periods may lead to physiological and genetic damage (Pilatti et al., 2010). The DNA damage, the risk of disease and plagues striking, besides the need to perform viability control, all typical of traditional storage, are reduced or eliminated all together with the use of seed cryoconservation (Tarré et al*.,* 2007).

Cryoconservation techniques have been much applied to long-term storage of seeds in germplasm banks (Lima et al., 2008). Cryoconservation is specially indicated for species of vegetative propagation, species with recalcitrant seeds, rare germplasm or even threatened species (Engelmann, 2011). However, few studies have been made with seeds of tropical climate (Lima et al., 2008), and the development of techniques for seed storage is fundamental in order to conserve genetic resources in germplasm banks and to promote the longevity of threatened species seeds (Vásques-Yanes and Orozco-Segovia, 1993; Raven and Havens, 2014).

The collecting of germplasm to ensure seed conservation is, on the other hand, of extreme importance on account of preserving the physiological quality of those seeds, maintaining genetic variability of threatened species and subsidizing reintroduction programs for extinct populations (Cabral et al., 2003; Lima et al., 2008; Guerrant et al., 2014). Technological studies of seeds are the actual starting point for mindful use and exploration of native species (Tresena et al*.,* 2010).

This article reports studies made in Brazil for cryoconservation of native plant species and highlights which might be the gaps in this area of germplasm conservation.

CRYOPRESERVATION OF NATIVE PLANTS

Brazil is among the most diverse countries in the world regarding plants and their genetic resources, and the species that occur there are used for food and beverages, medicinal and ornamental purposes, or have valuable wood. Many species are not cultivated, and therefore are threatened by illegal collection, and with the development and economic growth Brazil has been going through in the last few decades, urban and agricultural expansion is also a very serious threat to Brazilian genetic resources and environment preservation (Santos et al., 2013).

The development of protocols for the cryoconservation of native plants in Brazil remains limited, with most of the efforts directed to economically important species. Due to recalcitrance, limited supply of germplasm and lack of knowledge regarding the behavior of their seeds when stored, their adaptive physiology and their response to culture, groups of native and threatened species are not targeted for conservation studies often. *In vitro* manipulation and cryoconservation worksed with intermediary and orthodox seeds are the ones achieving the greatest advances in this area (Pilatti et al., 2010).

There have been made approximately 24 cryoconservation studies with Brazilian plant species, which are listed in Table 1. Only five of these works applied some kind of cryoprotector in order to achieve conservation of the plant material, which may be related to the fact that these studies used calli, apexes and buds instead of only seeds (Charoensuba et al., 2003; Nogueira, 2010; Rodrigues et al., 2012; Porto, 2013); the one that did use seeds was a study with an Orchidaceae species (Galdiano et al., 2013). The use of vitrification procedures prevents most of the ice crystal formation typical of traditional procedures (Engelmann, 2011), which is associated to cellular rupture, damage of organelles and bubble formation (Fuller, 2004). All the other studies found in literature used only seeds as conserved plant material and did not apply cryoprotectors to them, which in turn may be due to the toxicity of this kind of treatment (Fuller, 2004) or to the raise in protocol costs. It could also be an unnecessary step to add in a protocol if the results of cryoconservation of seeds without cryoprotectors are already effective.

Most of the species cited in Table 1 had their seeds classified as orthodox or intermediary, with only a few species of the Passifloraceae family being labeled as recalcitrant. The desiccation of the seeds was performed prior to freezing at the authors' criteria in each study, varying according to the targeted species. Since desiccation decreases water content within the seed, it tends to lower the probability of damage taking place, normally associated to the presence of water in the tissues during the process of cryoconservation. According to Engelmann (2004), the survival of the frozen samples is optimized when seeds are frozen with water content between 10 and 20%, which is in accordance with the majority of species listed in Table 1. Some species, however, were stored with a water content above this limit range, like, for instance, *Blepharocalyx salicifolius*, *Eugenia jambolana*, *Parapiptadenia rigida* (Venzke et al., 2006), *Encholirium scrutor* and *Dyckia ursina* (Tarré et al., 2007). This reiterates that the water content limit range for cryoconservation of seeds varies depending on the target species. On the other hand, according to Pritchard and Nadaranjan (2007), moisture contents ≤ 8% do not affect the survival of orthodox seeds after cryoconservation. This is also in accordance with many species listed in Table 1, such as *Astronium urundeuva* (Medeiros and Cavallari, 1992), *Spondays mombin*, *Byrsonima basiloba* (Salomão, 2002), *Styrax camporum* (Lima et al., 2008), and *Tabebuia chrysotrica* (Tresena et al., 2010).

Regarding recalcitrant seeds, it is estimated that 70% of tropical forest species have seeds that belong to this category (Barbedo et al., 2002), and is still necessary to establish specific protocols related to water content and desiccation tolerance. According to Pilatti et al. (2010), during the last few decades efforts have been made to expand knowledge of plants with recalcitrant seeds, but in the present study this was not found in literature regarding native species targeted for cryoconservation. The conservation of such seeds tends to be more problematic than the conservation of orthodox and intermediary seeds, which is due, sometimes, not only to their intolerance to desiccation, but due to difficult access to these plants for sample collection, the devastation of their habitat, and the irregularity of seed production throughout the year (Pilatti et al., 2010; Walters et al., 2013). All of these factors lead to lack of plant material available for conducting experiments, to lack of knowledge regarding the physiology and reproduction of these plants, and, finally, it is reflected in the shortage of studies about cryoconservation of species with recalcitrant seeds native to Brazil, as presented in this work.

Nonetheless, cryoconservation of recalcitrant seeds can be achieved using plant tissue culture technologies to isolate the embryo and germinate it *in vitro*, and with the use of cryoprotectors and the reduction of stress caused by free radicals, thus conserving the plant germplasm in liquid nitrogen (Walters et al., 2013; Pammenter and Berjak, 2014). Moreover, cryoconservation studies with orthodox seeds should be used to improve the development of protocols for the germplasm of intermediary and recalcitrant seeds of native Brazilian species (Pilatti et al., 2010).

Concerning plants that only reproduce vegetatively, there were no studies of cryoconservation found for native species of Brazil that fall into that category. Nevertheless, protocols have been established successfully for many ornamental species and species of agronomic importance, be it fruit-bearing trees, tubers or

Table 1. Family, water content (%), plant material used desiccation prior to storage, use of cryoprotection and time of storage of cryoconserved plant species native to Brazil.

Family	Species	Plant material	Water content (%)	Desiccation	Cryoprotection	Storage time	Reference
Anacardiaceae	*Anacardium othonianum*	Seed	12.0 - 14.0	Yes	No	20 days	Silva et al., 2013
Anacardiaceae	*Astronium fraxinifolium*	Seed	6.3	No	No	3 days	Salomão, 2002
Anacardiaceae	*Astronium urundeuva*	Seed	6	Yes	No	15 days	Medeiros et al., 1992
Anacardiaceae	*Astronium urundeuva*	Seed	9	No	No	5, 15 and 25 days	Gonzaga et al., 2003
Anacardiaceae	*Lithraea brasiliensis*	Seed	31	No	No	1 h	Venzke et al., 2006
Anacardiaceae	*Schinopsis brasiliensis*	Seed	6.7	No	No	3 days	Salomão, 2002
Anacardiaceae	*Schinopsis brasiliensis*	Seed	7.4	No	No	5, 15 and 25 days	Gonzaga et al., 2003
Anacardiaceae	*Spondays mombin*	Seed	4.1	No	No	3 days	Salomão, 2002
Anarcadiaceae	*Astronium fraxinifolium*	Seed	5.9	No	No	3 days	Lima et al., 2008
Anarcadiaceae	*Myracrodruon urundeuva*	Seed	12	No	No	3 days	Lima et al., 2008
Anarcadiaceae	*Schinopsis brasiliensis*	Seed	7.3	No	No	3 days	Lima et al., 2008
Apocynaceae	*Aspidorperma pyrifolium*	Seed	5.4	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma discolor*	Seed	5.7	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma parvifolium*	Seed	7.2	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma pyrifolium*	Seed	6.8	No	No	3 days	Lima et al., 2008
Apocynaceae	*Hancornia speciosa*	Apical buds/ calli	-	-	Yes	60 mins/24 h	Nogueira, 2010
Bignonaceae	*Tabebuia chrysotrica*	Seed	4	Yes	No	5 days	Tresena et al., 2010
Bignonaceae	*Tabebuia heptaphylla*	Seed	8	Yes	No	30, 60 and 90 days	Tresena et al., 2009
Bignonaceae	*Tabebuia umbellata*	Seed	6	Yes	No	7 days	Wetzel et al., 2003
Bignoniaceae	*Anemopaegma arvense*	Seed	6.3	No	No	3 days	Salomão, 2002
Bignoniaceae	*Jacaranda brasiliana*	Seed	5.7	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Jacaranda cuspidifolium*	Seed	8.5	No	No	3 days	Salomão, 2002
Bignoniaceae	*Jacaranda decurrens*	Seed	5.2	No	No	3 days	Salomão, 2002
Bignoniaceae	*Pithecoctenium squalus*	Seed	10.7	No	No	1 h	Venzke et al., 2006
Bignoniaceae	*Tabebuia aure*	Seed	6.7	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Tabebuia aurea*	Seed	7	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia impetiginosa*	Seed	5.8	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia impetiginosa*	Seed	7.5	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Tabebuia roseo-alba*	Seed	5.8	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia serratifolia*	Seed	5.4	No	No	3 days	Salomão, 2002
Bignoniaceae	*Zeyheria montana*	Seed	5.5	No	No	3 days	Salomão, 2002
Bombacaceae	*Cavanillesia arborea*	Seed	8.5	No	No	3 days	Lima et al., 2008
Bombacaceae	*Chorisia pubiflora*	Seed	8.5	No	No	3 days	Salomão, 2002
Bombacaceae	*Chorisia speciosa*	Seed	5.8	Yes	No	7 days	Wetzel et al., 2003
Bombacaceae	*Eriotheca gracilipis*	Seed	6.5	No	No	3 days	Salomão, 2002

Table 1. Contd.

Bombacaceae	*Pseudobombax cf. tomentosum*	Seed	7	No	No	3 days	Salomão, 2002
Boraginaceae	*Cordia trichotoma*	Seed	7.3	No	No	3 days	Lima et al., 2008
Bromeliaceae	*Dyckia sordida*	Seed	13.4	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Dyckia ursina*	Seed	28.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium heloisae*	Seed	19.1	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium magalhaesii*	Seed	14.6	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium pedicellatum*	Seed	2.5	Yes	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium reflexum*	Seed	11.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium scrutor*	Seed	24.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium subsecundum*	Seed	12.7	No	No	1 day	Tarré et al., 2007
Cactaceae	*Cereus jamacaru*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Discocactus zehntneri*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Discocactus zehntneri*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Cactaceae	*Melocactus albicephalus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus concinnus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus ernestii*	Seed	6.8	No	No	7, 34 and 120 days	Assis et al., 2011
Cactaceae	*Melocactus paucispinus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus zehntneri*	Seed	8.3	No	No	7, 34 and 120 days	Assis et al., 2011
Cactaceae	*Micranthocereus flaviflorus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Pilosocereus gounellei*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Pilosocereus gounellei*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Cactaceae	*Stephanocereus luetzelburgii*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Caesalpiniaceae	*Apuleia leiocarpa*	Seed	3.5	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia acuruana*	Seed	5.7	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia* sp.	Seed	6.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia ungulata*	Seed	5.2	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Chamaecrista desvauxii*	Seed	7.7	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Copaifera langsdorffii*	Seed	5.9	No	No	3 days	Lima et al., 2008
Caesalpiniaceae	*Dialium divaricatum*	Seed	8.4	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Dimorphandra mollis*	Seed	8.4	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Hymenaea courbaril var.stilbocarpa*	Seed	5.8	No	No	3 days	Lima et al., 2008
Caesalpiniaceae	*Melanoxylum brauna*	Seed	9.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Peltogyne confertiflora*	Seed	10.1	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Sclerolobium paniculatum*	Seed	6.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Senna alata*	Seed	7.2	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Senna* sp.	Seed	9.3	No	No	3 days	Salomão, 2002

Table 1. Contd

Caesalpinioideae	*Caesalpinia echinata*	Seed	-	-	No	30, 90 and 180 days	Zanotti et al., 2007
Caesalpinoideae	*Bauhinia* sp.	Seed	6.9	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Cassia ferruginea*	Seed	6.5	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Hymenae stignocarpa*	Seed	5	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Hymeneae courbaril*	Seed	-	No	No	7 days	Farias et al., 2006
Caesalpinoideae	*Sclerolobium aureum*	Seed	6.5	Yes	No	7 days	Wetzel et al., 2003
Combretaceae	*Buchenavia tomentosa*	Seed	8.1	No	No	3 days	Salomão, 2002
Cucurbitaceae	*Lagenaria vulgaris*	Seed	13	No	No	1 h	Venzke et al., 2006
Cucurbitaceae	*Luffa cylindrica*	Seed	12	No	No	1 h	Venzke et al., 2006
Dioscoreaceae	*Dioscorea* sp.	Seed	12.4	No	No	3 days	Salomão, 2002
Eriocaulaceae	*Syngonanhus arthrotrichus*	Seed	-	No	No	40, 80 and 120 days	Duarte, 2009
Eriocaulaceae	*Syngonanthus elegans*	Seed	-	No	No	40, 80 and 120 days	Duarte, 2009
Euphorbiaceae	*Jatropha curcas*	Seed	8	Yes	No	5 days	Goldfarb et al., 2008
Euphorbiaceae	*Jatropha curcas*	Seed	8.3	Yes	No	30, 60 and 90 days	Silva et al., 2011
Euphorbiaceae	*Manihot esculenta*	Axillary apices	-	No	Yes	2 h	Charoensuba et al., 2003
Euphorbiaceae	*Manihot esculenta*	Meristems	-	No	Yes	15 days	Rodrigues et al., 2012
Fabaceae	*Amburana cearensis*	Seed	5.3	No	No	3 days	Salomão, 2002
Fabaceae	*Amburana cearensis*	Seed	8.9	No	No	3 days	Lima et al., 2008
Fabaceae	*Anadenanthera colubrina*	Seed	7.1	No	No	3 days	Salomão, 2002
Fabaceae	*Bowdichia virgilioides*	Seed	5.9	No	No	3 days	Salomão, 2002
Fabaceae	*Crotalaria cf. spectabilis*	Seed	15	No	No	3 days	Salomão, 2002
Fabaceae	*Cyclolobium cf. blanchetianum*	Seed	6.8	No	No	3 days	Salomão, 2002
Fabaceae	*Dalbergia miscolobium*	Seed	8.6	No	No	3 days	Salomão, 2002
Fabaceae	*Lonchocarpus montanus*	Seed	5.3	No	No	3 days	Lima et al., 2008
Fabaceae	*Machaerium aculeatum*	Seed	4.2	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium brasiliensis*	Seed	4.9	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium cf. acutifolium*	Seed	6.4	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium scleroxylon*	Seed	8.5	No	No	3 days	Lima et al., 2008
Fabaceae	*Ormosia fastigiata*	Seed	3	No	No	3 days	Salomão, 2002
Fabaceae	*Platypodium elegans*	Seed	8.4	No	No	3 days	Salomão, 2002
Fabaceae	*Pterodon emarginatus*	Seed	5.3	No	No	3 days	Salomão, 2002
Fabaceae	*Stryphnodendron adstrigens*	Calli/shoot tips/seeds	6 - 9	Yes	Yes	1 h/1 h/24 h	Porto, 2013
Gramineae	*Euchlaena mexicana*	Seed	16	No	No	1 h	Venzke et al., 2006
Guttiferae	*Kielmeyera coriacea*	Seed	8.7	No	No	3 days	Salomão, 2002
Lecythidaceae	*Cariniana estrellensis*	Seed	5.9	No	No	3 days	Salomão, 2002
Lecythidaceae	*Cariniana legalis*	Seed	7.2	No	No	3 days	Salomão, 2002

Table 1. Contd.

Lytraceae	*Lafoensia pacari*	Seed	9.2	No	No	3 days	Salomão, 2002
Malpighiaceae	*Byrsonima basiloba*	Seed	3	No	No	3 days	Salomão, 2002
Meliaceae	*Cedrela fissili*	Seed	8	No	No	3 days	Lima et al., 2008
Meliaceae	*Cedrella fissilis*	Seed	9.8	No	No	3 days	Salomão, 2002
Meliaceae	*Cedrella fissilis*	Seed	-	No	No	1 h	Nunes et al., 2003
Mimosaceae	*Acacia farnesiana*	Seed	6.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Acacia polyphylla*	Seed	9.4	No	No	3 days	Lima et al., 2008
Mimosaceae	*Albizia* sp.	Seed	6.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Anadenanthera colubrina*	Seed	8.8	No	No	3 days	Lima et al., 2008
Mimosaceae	*Enterolobium contortisiliquum*	Seed	7.7	No	No	3 days	Salomão, 2002
Mimosaceae	*Enterolobium contortisiliquum*	Seed	7.3	No	No	3 days	Lima et al., 2008
Mimosaceae	*Enterolobium gummiferum*	Seed	7.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Mimosa somnians var. viscida*	Seed	12.2	No	No	3 days	Salomão, 2002
Mimosaceae	*Mimosa* sp.	Seed	7.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Parapiptadenia rigida*	Seed	35	No	No	1 h	Venzke et al., 2006
Mimosaceae	*Stryphnodendron polyphyllum*	Seed	5.1	No	No	3 days	Salomão, 2002
Mimosaceae	*Stryphnodendron pulcherrimum*	Seed	13	No	No	3 days	Salomão, 2002
Mimosoideae	*Albizia lebbeck*	Seed	6.1	Yes	No	7 days	Wetzel et al., 2003
Mimosoideae	*Anadenanthera macrocarpa*	Seed	5	Yes	No	7 days	Wetzel et al., 2003
Mimosoideae	*Mimosa setosa Benth*	Seed	3.5	Yes	No	7 days	Wetzel et al., 2003
Monimiaceae	*Siparuna guianensis*	Seed	6.4	No	No	3 days	Salomão, 2002
Myrsinaceae	*Myrsine laetevirens.*	Seed	16	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Blepharocalyx salicifolius*	Seed	32	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Eugenia jambolana*	Seed	52	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Psidium guajava*	Seed	7	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Psidium guajava L. v. pomifera*	Seed	11.7	No	No	1 h	Venzke et al., 2006
Orchidaceae	*Oncidium flexuosum*	Seed	11.3	No	Yes	30 min	Galdiano et al., 2013
Papilionoideae	*Platypodium elegans*	Seed	5.4	Yes	No	7 days	Wetzel et al., 2003
Passifloraceae	*Passiflora edulis*	Seed	22-34	Yes	No	10 days	Meletti et al., 2007
Passifloraceae	*Passiflora nitida*	Seed	30	Yes	No	10 days	Meletti et al., 2007
Passifloraceae	*Passiflora serrato-digitata*	Seed	21	Yes	No	10 days	Meletti et al., 2007
Polygonaceae	*Triplaris gardneriana*	Seed	5.6	No	No	3 days	Salomão, 2002
Proteaceae	*Roupala montana*	Seed	7.5	Yes	No	7 days	Wetzel et al., 2003
Rubiaceae	*Guettarda pohliana*	Seed	3.5	No	No	3 days	Salomão, 2002
Rubiaceae	*Tocoyena formosa*	Seed	5.9	No	No	3 days	Salomão, 2002
Sapindaceae	*Magonia pubescens*	Seed	5	No	No	3 days	Salomão, 2002
Sterculiaceae	*Sterculia striata*	Seed	11.1	No	No	3 days	Salomão, 2002

Table 1. Contd.

Sterculiaceae	*Sterculia striata*	Seed	10.9	No	No	3 days	Lima et al., 2008
Styracaceae	*Styrax camporum*	Seed	3	No	No	3 days	Salomão, 2002
Styracaceae	*Tiliaceae Luehea* sp.	Seed	7.5	No	No	3 days	Salomão, 2002
Tiliaceae	*Apeiba tibourbou*	Seed	6.9	No	No	3 days	Salomão, 2002
Verbenaceae	*Aegiphila lhotzkiana*	Seed	4.6	Yes	No	7 days	Wetzel et al., 2003
Vochysiaceae	*Qualea parviflora*	Seed	8.5	Yes	No	7 days	Wetzel et al., 2003

roots, of tropical or temperate origins, through the use of vitrification and other strategies (Engelmann, 2011). The same strategies of tissue cryoconservation available for plants with recalcitrant seeds could also be used for preserving the genetic diversity of plants that only reproduce vegetatively or that possess seeds or spores that traditional methods are unable to preserve *ex situ* (Pence, 2014).

In the next sections studies found in literature regarding cryoconservation of Brazilian plant species will be presented distributed in four categories, namely, Forest species, Medicinal and edible species, and Ornamental species. These categories are arbitrary, chosen for better exploration of contents, and many species might fit in more than one of them, despite being allocated here in the category representing their main usage by man. The complete list of works and species can be found in Table 1. A survey of the literature from 1992 to 2014 for studies regarding the cryoconservation of Brazilian native plant species was conducted. These works are available on the Internet and were obtained through searches in databases such as Scielo (Scientific Electronic Library Online), Google Scholar and "Portal de Periódicos CAPES/MEC" (http://www.periodicos.capes.gov.br/). To the best knowledge of the authors of this article, all of the papers regarding the cryoconservation of plant germplasm native to Brazil that were available for download are presented in the following sections.

Forest species

The capability to tolerate ultralow temperatures (-196°C) was tested for 66 tropical species, belonging to 21 botanical families of Cerrado and Atlantic Forest (Salomão, 2002). In 51 of these species germinability (germination percentage) was not affected by cryoconservation, and in nine of them there was an enhancement in germinability or a break of seed dormancy with freezing, while six showed significant reduction in germinability (Table 1).

In the Laboratory of Seed Physiology of Embrapa (Brazilian Agricultural Research Corporation) Genetic Resources and Biotechnology, the behavior of forest species seeds collected in the Brazilian Cerrado was evaluated after seven days of storage in liquid nitrogen (Wetzel et al., 2003). Seeds of thirteen species of seven different families were studied (Table 1). They were previously dehydrated in drying chamber (20±3°C and 12±3% of relative humidity). The initial average germination was 64.92%, and after the freezing period the average was 62.23%, which indicated that dry seeds of the studied species resisted the temperature of -196°C, suggesting that this technique can be used for the conservation of native forest species in germplasm banks (Wetzel et al., 2003).

Zanotti et al. (2007) evaluated the development of seedlings and saplings of pau-brasil (*Caesalpinia echinata*) originated from cryoconserved seeds. Regarding the initial development, the authors noted significant differences, although these differences diminished as the plants grew. According to the data obtained there were no significant differences between treatments, and plant growth was normal even for plants from up to 180 days of storage at -18 and -196°C (Zanotti et al., 2007).

Aiming to increase germination in the field for ecological restoration projects by direct seeding of dry forest tree species of the Paranã river basin (GO), Lima et al. (2008) investigated if seeds of such species alter their germinability after being stored in natural conditions for three to 15 months and in germplasm banks at -20 and -196°C. Storage at -20 and -196°C was efficient for preservation of seed physiological qualities for most of the arboreal species from deciduous forests of the Paranã river basin, being an alternative for *ex situ* conservation and for the increase of field germination in restoration projects by direct seeding (Lima et al., 2008).

The yellow ipê (*Tabebuia chrysotrica*) has seeds of short life spam, hindering its use in reforestation and the commercialization of its seedlings (Tresena et al., 2010). It was determined that the most appropriate water content limit for the cryoconservation of yellow "ipê" seeds

subjected to the temperature of -196°C, for five days, is 4%. No studies were made with longer periods of cryo-conservation of this species, but the data obtained by Tresena et al. (2010) established that the yellow “ipê” resists freezing and thawing with a maximum of 4% of water content in its seeds.

Medicinal and edible species

Medeiros and Cavallari (1992) conducted an experiment to analyze the germination of “aroeira” seeds *(Astronium urundeuva*), Anacardiaceae, after they had been dried and immersed in liquid nitrogen, in order to conserve the germplasm of this species in the Genetic Resources Conservation Area of Embrapa Genetic Resources and Biotechnology. This species is at risk of extinction because of the extraction of its wood, widely used in construction industry, and also for having medicinal use. The bark can be used to treat diseases of the airways and urinary tract, and its resin is applied for its tonic effect (Medeiros and Cavallari, 1992; Gonzaga et al*.,* 2003). The seeds were subjected to drying in a drying chamber (25°C and 10-15% of relative humidity) and, afterwards, samples were immediately conditioned in hermetic packages and immersed directly in liquid nitrogen (-196°C) for 15 days. The results indicate that “aroeira” seeds can be classified as orthodox and previously dehydrated up to 6% of water content to be directly stored in liquid nitrogen, and thus conserved by the cryoconservation method (Medeiros and Cavallari, 1992).

Gonzaga et al. (2003) studied not only the cryocon-servation of “aroeira”, but also of “baraúna” (*Schinopsis brasiliensis*), Anacardiaceae, another threatened species of the Brazilian semi-arid environment. Its wood is used for manufacture of furniture and in construction, but also for its digestive and analgesic action in popular medicine. Seeds were conserved in liquid nitrogen (-96°C) and in nitrogen vapor (-170°C), for a period of 25 days. The authors concluded that the seeds of both “aroeira” and “baraúna” can be cryoconserved in liquid nitrogen as well as in its vapor, and that “baraúna” seeds have their germinability increased when conserved in these temperatures for 25 days compared to control, which indicates that, during this time, seed dormancy break happens.

The “jatobá” (*Hymeneae courbaril*) is found in the Amazon Forest and the Brazilian Atlantic Forest, where it occurs naturally from the state of Piauí to the north of Paraná state. Its wood is also used for manufactoring furniture and in construction, and its fruits are consumed as food, and its leaves and seeds are utilized in the cosmetic and pharmaceutical industries (Farias et al*.,* 2006). “Jatobá” seeds from the tip and the middle section of the pod were subjected to different temperatures and it was concluded that the cryoconservation at -170°C showed higher germination rates and vigor (91% and 72%, respectively), being, thus, recommended (Farias et al., 2006).

The passionfruit (Passifloraceae) is a native plant broadly used in Brazilian alimentation, and at least a third of the *Passiflora* species has its center of origin in Brazil (Meletti et al., 2007). Seeds of six accessions of passion-fruit from the Active Germplasm Plant of the Agronomic Institute of Campinas (IAC) were subjected to cryocon-servation with or without previous desiccation, and different responses to storage in liquid nitrogen were observed (Meletti et al., 2007). The commercial varieties of *P. edulis* can be cryoconserved after desiccation, but *P. nitida* and *P. serrato-digitata* behaved as recalcitrant and intermediary seeds, respectively, and did not have their physiological quality improved by cryoconservation.

The physic nut (*Jatropha curcas*) is an oleaginous species with potential for biofuel production, found in the semi-arid regions of Brazil (Goldfarb et al., 2008; Silva et al., 2011). Its seeds were dehydrated in drying chamber and then rehydrated until the water content ranged between 4 and 14% for different treatments and were cryoconserved for five days, being gradually thawed at the end of the period (Goldfarb et al., 2008). The water content limit suggested for the species was 8%, but with 4% to 8% of humidity the seeds did not have their quality altered significantly. Seeds with 8% of water content were cryoconserved for 30, 60 and 90 days immersed in the nitrogen vapor or liquid nitrogen (-170 and -196°C, respectively), maintaining their vigor and viability at both temperatures and during all of the storage periods (Goldfarb et al., 2010). In another more recent study, seeds were dried with silica gel for 24 or 48 h, and then stored in liquid nitrogen for 60 and 90 days (Silva et al., 2011). In this case, cryoconserved seeds also maintained their viability high with moisture content close to 8%, but the desiccation combined with cryoconservation damaged cells and tissues, causing abnormalities in plants.

Seeds of “mangabeira” (*Hancornia speciosa*) have low germination rate and recalcitrance, which makes this Cerrado species difficult to conserve and multiply (Nogueira, 2010). Synthetic seeds made of apical buds from the “mangabeira” tree in a sodium alginate matrix were subjected to 60 mins in liquid nitrogen, but no regeneration was observed from such propagules after storage (Nogueira, 2010). The cryoconservation of mangabeira calli was achieved with previous dehydration followed by pre-treatment with cryoprotectors and immersion in liquid nitrogen for 24 h (Nogueira, 2010). The cryoconserved calli showed decline of cellular viability after 30 days of culture, and analysis identified plasmolyzed cells and residual cryoprotectors after cryoconservation.

The cassava (*Manihot esculenta*), a plant native to the Amazonas region, is estimated to have two thirds of its genetic diversity in *in situ* collections or natural habitat, with only one third being maintained *ex situ* (Santos et al., 2013). Recently, plant tissue culture methods have

been used to conserve and multiply accessions of cassava, as well as to assist in the cryoconservation of this species (Charoensuba et al., 2003; Rodrigues et al., 2012). Axillary apices of *M. esculenta* were cultured on medium supplemented with 0.3 M sucrose for 16 hours, followed by a treatment with 2 M glycerol and 0.4 M sucrose for 20 min for cryoprotection (Charoensuba et al., 2003). After 45 min in a solution of PVS2 and then two hs in liquid nitrogen, the apices were cultured and resumed growth, with a regeneration rate of 70% (Charoensuba et al., 2003). Other tests showed that meristems exposed to a vitrification solution containing 0.5% of Tween-20 were able to achieve an *in vitro* regeneration rate of 50% after cryoconservation (Rodrigues et al., 2012).

The "barbatimão" (*Styphnodendron adstringens*), also found in the Brazilian Cerrado, is rich in tannins and has medicinal applications (Porto, 2013). Calli obtained from cotyledon explants induced with 1 mg L^{-1} of 2,4-D (dichlorophenoxyacetic acid) in MS medium (Murashigue and Skoog, 1962) were cryoconserved, but after storage growth was not resumed in the cultures (Porto, 2013). Shoot tips cultured in medium with 0.25 mg L^{-1} of 6-benzylaminopurine (BAP) were exposed to PVS2 (30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide and MS medium with 0.4 M of sucrose) or modified PVS2 (40% glycerol, 10% ethylene glycol, 10% dimethyl sulfoxide and MS medium with 0.4 M of sucrose) for different periods of time and then cryoconserved. The exposure for 15 and 30 min followed by culture in medium with gibberelic acid (GA_3) and 2.0 mg L^{-1} of BAP obtained the highest survival rate and the longest shoot length, respectively (Porto, 2013). "Barbatimão" seeds were also cryoconserved by Porto (2013), and, with previous desiccation, seeds that originated seedlings with higher dry weight and length were those with moisture content between 6 and 9%.

The "caju-de-árvore-do-Cerrado" (*Anacardium othonianum*), another species from the Brazilian Cerrado, is appreciated for its fruits eaten *in natura* or as juice, liqueur and sweets, as well as for its roasted nut (Silva et al., 2013). Its seeds were cryoconserved by Silva et al. (2013) with the aim of testing the influence of water content and different thawing methods over its germination. The water content limit found was the range of 12 to 14%, being that lower contents caused decrease in germination, and most of the thawing methods tested did not interfere with seed viability, except the microwave, which caused an inferior germinative response.

Ornamental species

Tarré et al. (2007) studied the effect of desiccation, storage at low temperature and cryoconservation in eight species of Bromeliaceae, six of these from the genus *Encholirium* and two of the genus *Dyckia*, selected according to vulnerability and endemism criteria. The germinability after freezing in liquid nitrogen was higher or similar to the one obtained in the control for all species studied, but for *E. pedicellatum* tolerance to freezing was only achieved when seeds were dried until they contained 2,5% of water. Considering tolerance to low temperature storage and desiccation, seeds of these genera can be classified as orthodox and, according to the results obtained by Tarré et al. (2007), cryoconserved.

Similarly to bromeliads, "sempre-vivas" are exploited for their ornamental value and usage in decorations, which is why two of these commercialized species were cryoconserved by Duarte (2009): *Syngonanthus arthrotrichus* and *S. elegans*. They can be successfully cryoconserved for 40, 80 and 120 days and thawed at room temperature or in a microwave oven.

The wood of the pink "ipê" (*Tabebuia heptahylla*) is used for various purposes and the ornamental potential of this species is explored in landscaping and the afforestation of streets and avenues (Tresena et al., 2009). Its seeds can be cryoconserved, because the mean values of germinability and vigor were maintained after exposure for 90 days to the temperatures of -170 and -196°C (Tresena et al., 2009).

Cryoconservation by direct immersion of seeds in liquid nitrogen for 7, 30 and 120 days did not affect the germination of cacti native to Brazil with different degrees of threat and endemism (*Cereus jamacaru, Discocactus zehntneri, Melocactus xalbicephalus, M. concinnus, M. paucispinus, M. zehntneri, M. ernestii, Micranthocereus flaviflorus, Pilosocereus gounellei and Stephanocereus luetzelburgii*) (Veiga-Barbosa et al., 2010; Assis et al., 2011, Marchi et al., 2013). The cacti species investigated in these conservation studies are threatened by illegal trade that supplies the ornamental plant market, but also by the destruction and fragmentation of their habitat (Marchi et al., 2013). In the work reported by Assis et al. (2011), the seeds of *M. zehntneri* and *M. ernestii* showed higher germinability after cryoconservation when compared to control, which also happened to seeds of *D. zehntneri* after 30 days of storage in liquid nitrogen (Marchi et al., 2013). An advantage for cryoconservation of seeds of these cacti species is the characteristically low moisture content of orthodox seeds. Besides, another favorable feature for cactus conservation is the small dimension of the seeds of such species, including the ones of the threatened genera *Melocactus* and *Discocactus*.

The orchid *Oncidium flexuosum* is native to Brazil, Paraguay and Argentina, and it is used for its ornamental and medicinal qualities (Galdiano et al., 2013). In order to cryoconserve this species, cryoprotectors were added to the vitrification solution, and it was observed that vitrification using PVS2 for 120 mins with 1% of phloroglucinol is the best alternative to cryoconserve mature seeds of *O. flexuosum* (Galdiano et al., 2013).

This treatment enhanced the *in vitro* germination of the species by 68% and the plants originated showed normal development and no ploidy level changes (Galdiano et al., 2013).

CRYOBANKS IN BRAZIL

For cryoconservation to be effective, germplasm banks need to work with hundreds of thousands of accessions, which can only be achieved with high efficiency approaches and cryoconservation technical knowledge. In order to do so, the development of equipment and installations, control of the sampling process and of the manipulation of accessions, creation of inventory and database, quality control, standardization of industry practices, presence of biosecurity systems and the valorization of germplasm are all necessary (Varga and Tiersch, 2012).

When installing cryobanks, differences between cryoconservation methodologies must be avoided among different institutions so that a general germplasm bank may be formed to improve conserved material exchange. Thus, comparative validations of methods between different laboratories or cryoconserved species must be made, as well as economic analysis. The duplication of cryoconserved resources is advised, to limit the risk of loss (Keller et al., 2008).

According to Santos and Salomão (2010), the Embrapa Genetic Resources and Biotechnology has a cryobank for storage of a great number of samples in the facility where collections are stored, where there is a cryotank of great capacity, besides installations in which to perform experiments and establish protocols for various materials. Efforts have been made in the last two decades in order to cryoconserve native plant species, cultivated or not, that were not present in the traditional seed banks and collections (Santos et al., 2013), but most efforts have been targeting species of economic interest.

The Embrapa Genetic Resources and Biotechnology (previously known as National Centre of Genetic Resources - CENARGEN) is the main institution in Brazil that deals with the conservation of genetic resources (Embrapa, 2014), and cryoconservation is one of the goals for long-term conservation in this corporation. The company plans on building cryobanks to be used as base collections, which should include samples of species that are already stored in active collections, as well as species or products that are not yet stored in any bank or collection (Pádua, personal communication, 2014). Species with non-orthodox seeds, which will have their vegetative structures stored, and also species with orthodox seeds, will be cryoconserved in these cryobanks.

Periodic evaluations of genetic stability and biological integrity of the conserved material are recommended, although there are no protocols regarding which would be the ideal time interval for monitoring (Santos and Salomão, 2010). Information is necessary about the minimum acceptable levels of viability, the adequate sample size for each accession, the periodicity of monitoring and the effect of cryogenic protocols and regeneration over genetic stability of the material kept in liquid nitrogen (Santos, 2000).

Aiming to cryogenically store seeds of importance to biodiversity and of economic relevance in semi-arid region of Brazil, Cavalcanti et al. (2011) proposed a modeling for a cryogenic germplasm bank which included traceability of stored seeds. The software BCSeed (applied to management and control of seeds) was created with satisfactory performance in all topics in which it was structured after tests with various products (Cavalcanti et al., 2011).

A GLOBAL VIEW

Plant rarity is increasing worldwide and many species that need protection are not given proper attention not only because they are underappreciated but also conservation projects are often underfunded or neglected (Havens et al., 2014). Nevertheless, throughout the world, countries are concerned with environmental preservation and conservation, and many have been making efforts to safeguard the genetic resources of their native plant species for decades, even though, very similarly to Brazil, many are still establishing cryobanks and cryoconservation protocols.

Theres is lack of research on seed storage biology and germination, propagation and acclimatization protocols for Eastern Australian rainforest species, which have yet to be cryoconserved since few studies have applied *in vitro* and cryopreservation techniques to the conservation of these species (Ashmore et al., 2011). Research and projects are underway to improve the *ex situ* conservation of Eastern Australian rainforest species (Ashmore et al., 2011), as much as for most of the Brazilian flora. South Africa is also progressing in cryostorage research and its application through major efforts made by the authorities to achieve cryoconservation of native plant species, endangered or of economic importance (Berjak et al., 2011). The exchange of knowledge between Brazil and these countries could benefit germplasm conservation efforts in all parties involved.

In the United States of America, native and threatened plant species are being studied, documented and safely stored in seed banks, and the country has the largest somatic tissue culture collection of rare species in the world stored in liquid nitrogen through the cryogenic conservation program at Kings Park and Botanic Garden (Havens et al., 2014). In the Russian Academy of Sciences (Moscow), 15 species of rare medicinal plants and crops are conserved in liquid nitrogen through cell strains and seeds of 230 endangered plant species collected

collected in the Russian territory are cryoconserved, as well as seeds from 22 rare tropical and Russian orchids (Popov et al., 2006). Both of these countries are more advanced in cryoconservation of native plant germplasm than Brazil, and Brazilian institutions could also benefit from collaborations with their research groups and institutions.

In Latin America and the Caribbean, Brazil is one amongst 34 countries that are considered megadiverse regarding the biodiversity that exists in their territory (González-Arnao and Engelmann, 2013). The cryoconservation progress and development of this region of the world has been documented since studies started in the period of 1988-1990, with the professional training of researchers (González-Arnao and Engelmann, 2013).

Since then Latin American national institutions have developed projects with advanced technologies to increase knowledge regarding establishment and optimization of cryoconservation protocols, but also to verify stability of the conserved material (González-Arnao and Engelmann, 2013). The collaboration among national institutions and universities where cryoconservation research is performed in Latin America and the Caribbean is encouraged in order to establish cryobanks in the member countries and further research and genetic preservation of resources (González-Arnao and Engelmann, 2013).

CONCLUSIONS

More studies are necessary to better comprise the Brazilian biodiversity in regards to the cryoconservation of its genetic resources. Most studies use seeds as plant material, which means that species that do not propagate through seeds, or whose seeds are difficult to obtain, are not being prioritized because they are more difficult to study or have protocols established for them, and recalcitrant seeds can be highlighted in this aspect. Many of the works here addressed studied species threatened by anthropogenic activities and chose cryoconservation as a way to safeguard their germplasm. This calls attention to the threats which species native to Brazil are suffering and how much genetic resources may be lost if there is no intervention in favor of their conservation. The Brazilian cryobanks, however, seem to be in a state of shaping and development, with many studies yet to be made and many accessions yet to be incorporated to collections in order for them to achieve an ideal stage of operation and collaboration with other institutions worldwide.

Conflict of Interests

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

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Analysis of eleven Y-chromosomal STR markers in Middle and South of Iraq

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Samples of 320 random healthy unrelated Iraqi male persons from the Arab ethic group of Iraqi population were collected for Y chromosomal STRs typing. FTA® Technology was utilized to extract DNA from blood collected on FTA™ paper. We analyzed 11 Y chromosomal STR loci for evaluating allele frequencies and gene diversity for each Y-STR locus of the Y-Plex™ PCR amplification kit. The 11 loci include DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b. A total of 299 unique haplotypes was identified among the 320 individuals studied. The DYS385b had the highest diversity (GD = 0.8392), while loci DYS392 had the lowest (D = 0.2695). The study focuses to establish the basic forensic genetic informations, knowledge, data and statistics which might be so ultimately helpful practically in forensic science and criminology and to let evaluate and present the DNA weight evidences in Iraq medico-legal institute and courts of law.

Key words: Allele frequency, gene diversity, Iraq, STR DNA typing, Y-Plex™.

INTRODUCTION

The Y chromosome is constructed in males and includes the sex determining region and known as a paternal lineage marker (Butler et al., 2002; Kuppareddi et al., 2010; Carolina et al., 2010). Chromosome Y microsatellites or short-tandem repeats (STR's) seem to be ideal markers to delineate differences between human populations because they are transmitted in uniparental (paternal) fashion without recombination, also they are very sensitive for genetic drift, and they allow a simple highly informative haplotype construction (Kayser et al., 1997). The genetic information is inherited from the father to the son, and this information does not change except for mutational events (Hanson et al., 2007). These markers can also be useful in missing persons investigations, historical investigations, some paternity testing scenarios, and genetic genealogy (Park et al., 2007; Verzeletti et al., 2008).

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Unlike autosomal STR markers, Y-STR markers are linked on the same chromosome and there is no genetic recombination between the markers. Therefore, unlike for autosomal STRs, the Hardy-Weinberg equation is not suitable for determining the frequency of a genotype from the frequency of the alleles at each locus (Beleza et al., 2003; Dupuy et al., 2004). To determine the frequency of a particular Y-STR profile, the profile must be searched against different databases for a possible match, and these databases must be large enough to accurately represent the frequencies of the haplotypes present in the population of interest (Ballantyne et al., 2010).

For a number of forensic applications, Y-STR's could be superior to autosomal STR's, especially in rape cases where: (i) the differential lysis was unsuccessful, (ii) the number of sperm cells is very low, (iii) due to vasectomy epithelial cells instead of sperm cells from the ejaculate of the perpetrator have to be analyzed, and (iv) the perpetrator, due to a familial relationship shares many autosomal bands with the victim; Y-STR's could provide crucial evidence.

The Y chromosome is becoming a useful tool for tracing human evolution through male lineages (Jobling, 1995) as well as application in a variety of forensic situations (Kayser et al., 1997) including those involving evidence from sexual assault cases containing a mixture of male and female DNA (Prinz et al., 1997; Prinz et al., 2001). Using Y-chromosome specific methods can improve the chances of detecting low levels of male DNA in a high background of female DNA.

Ideally, a forensic Y chromosome haplotype should include as many polymorphic loci as possible to improve the chance of excluding individuals (or male lineages) who did not commit the crime. In order to obtain a high level of discrimination either a large number of Y STRs can be run one at a time or combined into a multiplex.

For Y STR systems to become more highly discriminatory and gain acceptance within the forensic, DNA community robust multiplexes are required. Y STR multiplexes have been described previously (Prinz et al., 1997; Redd et al., 1997; Gusmao et al., 1999).

MATERIALS AND METHODS

Preparation of blood stain samples

Blood samples were randomly collected from 320 unrelated Iraqi male persons from the Arab ethnic group of Iraqi population for DNA Y- chromosomal STRs typing; those samples were sent to the forensic genetic laboratories.

DNA extraction

DNA was extracted from all dried blood samples on FTA cards (Mullen et al., 2009; Dobbs et al., 2002) following the manufacturer's procedure as described in Whatman FTA Protocol BD01 except that the Whatman FTA purification reagent was modified to half the volume. A 1.2 mm diameter disc was punched from each FTA card with a puncher. The discs were transferred to new eppendorf tubes and washed three times in 100 µl Whatman FTA purification reagent. Each wash was incubated for 5 min at room temperature with moderate manual mixing and the reagent was discarded between washing steps. The discs were then washed twice in 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0); the buffer was discarded and the discs were left to dry at room temperature for 1 h.

Controls

It is recommended that internal standard controls are used during each PCR analysis which include the following: negative control, negative control with washed, no-sample punch, to ensure that the punch does not cause a positive result, positive control of a known DNA standard solution and positive control standard added to a normally washed, no-sample punch, to ensure that the punch does not inhibit the reaction.

DNA amplification for Y- Chromosomal STR

PCR is the process used to amplify a specific region of DNA. It is possible to create multiple copies from the small amount of template DNA using this process. 12Plex amplification was performed by the commercial kit Y-Plex™12 (Reliagene, New Orleans, LA) that amplifies 11 Y-STR loci (DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b) and a segment of the amelogenin gene, according to manufacturer's instructions but in a total reaction volume of 25 µl.

PCR amplicon analysis (capillary electrophoresis)

The major application of CE in forensic biology is in the detection and analysis of short tandem repeats (STRs). STR markers are preferred because of the powerful statistical analysis that is possible with these markers and the large databases that exist for convicted offenders' profiles. Using the ABI Prism1 3730xl Genetic Analyzer 16-capillary array system (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols, with POP-7™ Polymer and Data Collection Software, Genemapper version 3.5 software (Applied Biosystems), the allele designations were determined by comparison of the PCR products with those of allelic ladders provided with the kit. Nomenclature of loci and alleles is according to the International Society of Forensic Genetics (ISFG) guidelines reported by Gill et al. (2001). By comparison of the size of a sample's alleles to size of alleles in allelic ladders for the same loci being tested in the sample, the STR genotyping was conducted.

Quality control

Allelic ladders, male DNA (positive internal control), female DNA (negative control) and the amelogenin (internal control), provided by Reliagene (Reliagene Tech.) were used in each reaction with the Y-Plex™12 kit.

Statistical analysis for Y- chromosomal STR

Analysis of data

Allele frequencies were calculated by direct counting.

Allele diversity (genetic diversity) (D)

Allele diversity was calculated as (Nei, 1987):

$$D = \frac{n}{n-1}\left(1 - \sum_{i=1}^{n} p_i^2\right)$$

Where, n is the sample size and pi is the frequency of the ith allele.

Standard Error (SE)

The standard error (SE) of allele frequencies was calculated as:

$$SE(p_i) = \sqrt{[(1-p_i)p_i]/N},$$

Where, pi denotes the frequency of the *ith* allele at any given locus and N equals the total number of individuals screened at this locus.

RESULTS AND DISCUSSION

Y-chromosome (short tandem repeat) haplotypes, haplotype frequency, allele frequency and genetic diversity

Y-STR-Allele frequency and genetic diversity

Allelic genotyping of STRs does not require the use of complex molecular techniques, since amplifications and visualization of PCR products make it easy. Y-chromosome specific STRs (Y-STRs) are chosen as more informative in paternity testing, forensic applications and the study of population histories due to the haploid state of Y chromosome which ensures both the transmittance by the paternal lineages and the lack of recombination in NRY, excluding pseudoautosomal regions (PARs) (Betz et al., 2001; Corach et al., 2001; Dekairelle et al., 2001; Gill et al., 2004; Honda et al., 2001; Jobling et al., 1997). Allelic and haplotypic distributions of Y-STRs have shown significant differences in different geographical regions, ethnical groups and communities (Alves et al., 2003; Gusmao et al., 2003; Isobe et al., 2001; Rodig et al., 2007; Rustamov et al., 2004; Yan et al., 2007). Therefore, allelic and haplotypic frequencies of Y-STRs should be determined in a male population prior to any interpretations of forensic analysis and paternity testing (Budowle et al., 2003; Park et al., 2007). In this study, allelic and haplotypic frequencies involving 11 Y-STR loci have been determined with such a necessity in a representative group of Iraq population in order to make comparisons with other populations (Imad et al., 2013). Observed allele or genotype frequencies of the 11 Y-STR loci are given in Tables 1 and 2.

The DYS385b and DYS389II had the highest diversity (D = 0.8392 and 0.7140, respectively), while loci DYS392 and DYS439 had the lowest (D = 0.2695 and 0.2991, respectively). The frequencies of DYS392 were low in all allele except allele 11 where it was 85%; this allele is to be used to discriminate Iraqi men because of overall low genetic diversity, then this locus should be excluded because 85% of Iraqi men will have the locus. However it will be useful for discriminating Iraqi men from non-Iraqi men provided it is unique to only Iraqi men.

In another study on 17 Y-STR Y-chromosomal short tandem repeat loci from the Cukurova region of Turkey, the DYS391 recorded lowest gene diversity in this region which was 0.51 and the highest as 0.95 for DYS385a/b and no significant differences were found when this data was compared with haplotype data of other Turkish populations (Ayse et al., 2011). In Northern Greece the haplotype diversity was 0.9992 in 11 Y STR loci typed in a population sample of unrelated male individuals. Haplotypes were presented for the following loci: DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y GATA H4, DYS437, DYS438 and DYS448. This database study provides additional information for the application of Y chromosomal STRs to forensic identification efforts in Greece (Leda et al., 2008).

Y-STR- haplotypes and haplotype frequency

We identified 276 different haplotypes in our study sample (320 unrelated males), 299 of which (93.4%) were unique, six were found twice (2.8%) and six were found in three individuals (1.9%). The most frequent haplotype was haplotype number 78 (Table 2). Haplotype 78 seems to be specific to Iraq. This is to be corroborated by future investigations. The observed number of haplotypes and their frequencies has been tabulated in Table 3. We identified 96 different haplotypes in our study sample 94 of which (97.9%) were unique, one was found twice and one was found in three individuals. The most frequent haplotype was haplotype number 77. Haplotype 77 seems to be specific to Iraq. This is to be corroborated by future investigations. Haplotypes detected in this study group have been compared with seven other populations: German (n = 88), Indian (n = 25), Chinese (n = 36), Italians (n = 100) (Manfred et al., 2001), Tunis (n = 105) (Imen et al., 2005) and India (n = 154) (Kuppareddi et al., 2010) (Table 4). Haplotypic comparisons have highlighted significant differences from Iraq population in this study ($p < 0.05$). Our data have also provided additional information to the framework of variation involving 17 Y-STR loci as well as a further contribution to the Y-STR database for Iraq population. This supports the observations, by others (Jorde et al., 2000), that, especially among European populations, Y STRs are very powerful in the detection of genetic differences

Table 1. Allele frequencies and genetic diversity of (DYS19, DYS385a, DYS385b, DYS389I, DYS389II and DYS390) Y-STR loci in 320 Iraq males.

Allele	DYS19		DYS385a		DYS385b		DYS389I		DYS389II		DYS390	
	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	0.1625	0.021	-	-	-	-	-	-	-	-
12	-	-	0.0438	0.011	-	-	0.2750	0.024	-	-	-	-
13	0.3594	0.026	**0.5219**	0.028	0.0625	0.013	0.3406	0.026	-	-	-	-
14	**0.4219**	0.027	0.1719	0.021	**0.2500**	0.024	**0.3844**	0.027	-	-	-	-
15	0.1625	0.021	0.0094	0.005	0.1063	0.017	-	-	-	-	-	-
16	0.0343	0.010	0.0188	0.008	0.1438	0.021	-	-	-	-	-	-
17	0.0218	0.008	0.0563	0.013	0.0438	0.011	-	-	-	-	-	-
18	-	-	-	-	0.1688	0.021	-	-	-	-	-	-
19	-	-	0.0156	0.005	0.1719	0.021	-	-	-	-	-	-
20	-	-	-	-	0.0531	0.013	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	0.0500	0.011
22	-	-	-	-	-	-	-	-	-	-	0.0594	0.013
23	-	-	-	-	-	-	-	-	-	-	**0.4406**	0.028
24	-	-	-	-	-	-	-	-	-	-	0.3688	0.027
25	-	-	-	-	-	-	-	-	-	-	0.0813	0.014
28	-	-	-	-	-	-	-	-	0.0906	0.016	-	-
29	-	-	-	-	-	-	-	-	**0.4125**	0.027	-	-
30	-	-	-	-	-	-	-	-	0.2938	0.024	-	-
31	-	-	-	-	-	-	-	-	0.1188	0.018	-	-
32	-	-	-	-	-	-	-	-	0.0500	0.011	-	-
33	-	-	-	-	-	-	-	-	0.0344	0.010	-	-
GD*	0.6650		0.6547		0.8392		0.6606		0.7140		0.6573	

In **bold** are the most common allele for each locus. Freq, frequency; SE, standard error.

Table 2. Allele frequencies and genetic diversity of (DYS391, DYS392, DYS393, DYS438, and DYS439) Y-STR loci in 320 Iraq males.

Allele	DYS391		DYS392		DYS393		DYS438		DYS439	
	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE	Freq.	SE
8	0.0563	0.013	-	-	-	-	-	-	-	-
9	0.2188	0.024	-	-	-	-	0.1063	0.07	0.0094	0.005
10	**0.4969**	0.028	-	-	-	-	**0.6594**	0.024	**0.4094**	0.027
11	0.1250	0.018	**0.8500**	0.020	-	-	0.1281	0.018	0.3281	0.026
12	0.1031	0.018	0.0438	0.011	-	-	0.1063	0.017	0.0594	0.013
13	-	-	0.0688	0.013	0.1844	0.021	-	-	0.1250	0.018
14	-	-	0.0375	0.010	**0.6594**	0.024	-	-	0.0688	0.013
15	-	-	-	-	0.1313	0.021	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-

Table 2. Contd.

22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-
GD*	0.5802		0.2695		0.5140		0.5262		0.2991	

In **bold** are the most common allele for each locus. Freq, frequency; SE, standard error.

Table 3. Haplotypes for the 11 Y-STR loci observed 320 Iraq males.

Haplotype	DYS19	DYS385a	DYS385b	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS438	DYS439	N	F
H1	13	11	16	12	30	23	10	11	13	10	13	4	0.0125
H2	13	11	16	12	30	23	8	11	13	9	13	1	0.0031
H3	13	11	16	12	31	23	8	11	12	9	10	1	0.0031
H4	13	11	14	12	31	23	8	11	13	11	10	1	0.0031
H5	13	13	14	12	31	23	10	11	13	10	10	1	0.0031
H6	13	13	14	12	28	23	10	11	13	10	13	1	0.0031
H7	13	13	14	12	28	23	9	11	14	12	13	1	0.0031
H8	13	13	14	12	28	23	10	11	13	12	10	1	0.0031
H9	13	13	14	12	29	23	10	11	13	12	10	1	0.0031
H10	13	13	14	12	29	23	8	11	14	12	11	3	0.0094
H11	13	13	14	12	29	23	9	11	14	10	11	1	0.0031
H12	13	13	14	14	29	23	10	11	12	11	11	1	0.0031
H13	13	13	14	14	29	23	10	11	13	10	11	1	0.0031
H14	13	13	14	13	29	23	10	11	14	10	10	1	0.0031
H15	13	13	18	13	29	23	10	11	14	12	10	1	0.0031
H16	13	13	18	13	28	23	8	11	13	12	13	1	0.0031
H17	13	13	18	13	28	23	8	11	13	12	10	1	0.0031
H18	13	13	16	13	28	23	10	11	13	12	10	1	0.0031
H19	13	14	16	13	31	23	10	11	13	10	10	1	0.0031
H20	13	14	16	12	31	23	10	11	13	10	10	1	0.0031
H21	13	11	16	12	31	23	10	11	12	10	10	1	0.0031
H22	13	11	13	12	28	23	10	11	12	10	10	1	0.0031
H23	13	11	13	12	28	23	10	11	14	9	10	1	0.0031
H24	13	11	13	12	30	23	10	11	14	10	10	1	0.0031
H25	13	11	18	12	30	23	10	11	13	10	10	1	0.0031
H26	13	11	14	12	30	25	10	11	13	10	10	1	0.0031
H27	13	11	14	12	32	23	10	11	13	10	10	1	0.0031
H28	13	11	18	12	33	23	10	11	13	10	10	1	0.0031
H29	13	13	18	14	30	21	10	11	13	10	10	1	0.0031
H30	13	13	18	14	29	24	10	11	13	10	10	1	0.0031
H31	13	13	18	13	29	24	10	11	12	10	11	1	0.0031
H32	13	13	18	14	29	25	10	11	13	10	11	1	0.0031
H33	13	13	18	14	29	21	10	11	13	10	11	3	0.0094

Table 3. Contd.

H34	13	13	18	14	29	23	10	11	13	10	11	1	0.0031
H35	13	13	18	13	29	23	10	11	13	10	11	1	0.0031
H36	13	13	18	12	29	23	10	11	14	10	11	1	0.0031
H37	13	13	18	14	29	24	10	11	13	10	11	1	0.0031
H38	13	13	16	13	30	23	10	11	13	10	11	1	0.0031
H39	13	13	19	13	28	23	10	11	13	10	11	1	0.0031
H40	13	13	19	13	30	24	10	11	13	10	11	1	0.0031
H41	13	13	19	13	32	21	10	11	13	10	11	1	0.0031
H42	13	13	19	13	32	25	10	11	12	10	11	1	0.0031
H43	13	13	14	13	28	25	10	11	12	10	11	1	0.0031
H44	13	13	14	13	29	25	10	11	12	10	11	1	0.0031
H45	13	12	14	13	32	25	10	11	13	10	12	1	0.0031
H46	13	12	14	13	30	25	10	11	13	10	12	1	0.0031
H47	13	12	14	13	28	23	10	11	12	10	12	1	0.0031
H48	13	12	14	13	28	23	10	11	14	10	12	1	0.0031
H49	13	12	14	12	28	24	10	11	13	10	12	1	0.0031
H50	13	12	14	12	30	24	10	11	12	10	10	1	0.0031
H51	13	11	14	14	30	24	10	11	12	9	10	1	0.0031
H52	13	11	14	14	30	25	10	11	12	10	13	1	0.0031
H53	13	11	14	13	30	23	10	11	12	10	14	1	0.0031
H54	13	11	14	12	30	23	10	11	13	10	10	1	0.0031
H55	13	11	14	14	30	24	11	11	13	10	13	1	0.0031
H56	13	11	19	13	30	24	11	11	13	10	12	1	0.0031
H57	13	11	16	12	30	24	11	11	14	11	10	1	0.0031
H58	13	11	16	14	30	24	11	11	13	9	10	1	0.0031
H59	13	11	15	13	32	22	8	11	14	10	10	2	0.0063
H60	13	11	15	13	32	24	10	11	13	10	10	1	0.0031
H61	13	11	15	13	30	25	10	11	13	12	10	1	0.0031
H62	13	11	15	12	30	25	10	11	13	11	10	1	0.0031
H63	13	11	15	14	30	25	9	11	14	11	9	1	0.0031
H64	13	11	15	14	32	25	10	11	14	11	10	1	0.0031
H65	13	11	15	14	29	25	10	11	13	10	9	1	0.0031
H66	13	11	15	14	30	23	8	11	13	10	9	1	0.0031
H67	13	11	15	14	30	23	10	11	12	10	14	1	0.0031
H68	13	11	15	14	30	24	9	11	13	10	13	2	0.0063
H69	13	11	18	13	30	24	9	11	13	9	10	1	0.0031
H70	13	11	18	14	29	24	9	11	13	12	10	1	0.0031
H71	13	11	18	12	29	24	9	11	13	11	10	1	0.0031
H72	13	11	18	12	29	23	12	11	13	11	10	1	0.0031
H73	13	11	19	12	29	23	10	11	13	11	10	1	0.0031
H74	13	11	19	12	29	22	10	11	13	11	11	1	0.0031
H75	13	11	19	12	29	23	9	11	13	11	11	1	0.0031
H76	13	11	14	12	29	23	9	11	13	10	11	1	0.0031
H77	13	11	14	12	29	24	11	11	13	10	11	1	0.0031
H78	13	11	14	13	29	24	10	11	13	12	11	6	0.0188
H79	13	11	14	12	30	24	10	11	13	10	11	1	0.0031
H80	13	11	14	14	30	24	9	11	13	10	14	1	0.0031
H81	13	11	14	14	30	23	11	11	13	10	14	1	0.0031
H82	13	16	13	14	30	25	10	11	13	10	10	1	0.0031
H83	13	16	13	12	30	25	10	11	13	9	10	1	0.0031

Table 3. Contd.

H84	13	16	13	12	30	25	10	11	13	10	10	1	0.0031
H85	13	14	13	12	30	24	9	11	13	10	10	1	0.0031
H86	13	14	17	12	30	24	11	11	13	10	14	1	0.0031
H87	13	14	17	12	29	24	12	11	13	10	14	1	0.0031
H88	13	14	20	12	29	24	11	11	13	11	12	1	0.0031
H89	13	14	20	12	29	25	9	11	13	11	13	1	0.0031
H90	13	14	20	12	29	24	10	11	13	9	13	1	0.0031
H91	13	14	18	12	29	24	10	11	13	10	13	1	0.0031
H92	13	14	19	12	29	24	12	11	13	12	13	1	0.0031
H93	13	14	19	12	29	24	10	11	13	10	13	1	0.0031
H94	13	14	16	12	29	23	10	11	13	11	13	1	0.0031
H95	13	14	16	12	29	23	9	11	13	10	13	1	0.0031
H96	13	14	16	12	29	23	8	11	13	10	14	1	0.0031
H97	13	14	16	12	29	23	12	11	13	10	10	1	0.0031
H98	13	14	16	14	29	21	12	11	13	11	10	1	0.0031
H99	13	14	16	14	29	22	8	11	13	11	11	1	0.0031
H100	13	13	16	14	29	22	10	11	13	11	13	1	0.0031
H101	13	13	16	14	29	25	10	11	13	11	12	1	0.0031
H102	13	13	16	14	32	23	10	11	13	10	12	1	0.0031
H103	14	13	16	14	28	22	10	13	13	10	12	1	0.0031
H104	14	13	16	14	33	23	10	13	13	10	11	1	0.0031
H105	14	13	16	14	28	25	10	11	13	10	11	1	0.0031
H106	14	13	19	14	28	25	10	11	13	10	14	1	0.0031
H107	14	13	15	14	28	23	10	11	13	10	14	1	0.0031
H108	14	13	19	14	28	23	10	11	13	10	14	1	0.0031
H109	14	13	20	14	32	23	10	12	13	10	11	2	0.0063
H110	14	13	20	13	32	23	10	11	13	10	11	1	0.0031
H111	14	14	20	13	29	23	10	11	13	10	11	1	0.0031
H112	14	14	13	13	29	23	10	11	13	10	11	1	0.0031
H113	14	14	13	13	29	25	10	13	13	10	11	1	0.0031
H114	14	14	13	13	31	23	10	13	13	10	11	1	0.0031
H115	14	14	13	12	31	24	10	13	13	10	11	1	0.0031
H116	14	14	13	13	31	25	10	12	13	10	11	1	0.0031
H117	14	14	19	14	30	23	10	11	13	12	10	2	0.0063
H118	14	14	18	12	30	23	10	11	13	9	10	1	0.0031
H119	14	14	16	12	30	23	10	11	13	9	13	1	0.0031
H120	14	14	18	12	30	23	10	12	13	10	11	1	0.0031
H121	14	14	18	12	30	23	9	11	14	10	11	1	0.0031
H122	14	14	18	12	30	23	11	11	12	10	11	1	0.0031
H123	14	14	18	14	30	23	11	11	14	10	11	1	0.0031
H124	14	14	18	13	30	23	11	12	14	10	11	1	0.0031
H125	14	14	18	13	30	23	10	14	14	10	13	1	0.0031
H126	14	14	14	13	28	23	10	11	13	10	10	2	0.0063
H127	14	14	14	13	29	23	10	14	13	9	10	1	0.0031
H128	14	14	14	13	30	24	12	11	13	9	10	1	0.0031
H129	14	14	14	14	28	24	12	11	12	9	10	1	0.0031
H130	14	14	14	14	28	25	12	13	13	9	12	1	0.0031
H131	14	14	14	12	28	24	11	13	13	9	13	1	0.0031
H132	14	14	14	14	31	24	11	14	13	12	10	1	0.0031
H133	14	14	14	14	31	22	11	11	14	10	10	1	0.0031

Table 3. Contd.

H134	14	14	14	14	28	22	9	11	12	10	10	1	0.0031
H135	14	14	14	13	28	23	9	11	12	12	11	1	0.0031
H136	14	14	14	13	29	24	9	11	12	11	11	1	0.0031
H137	14	16	14	13	29	24	10	11	12	10	11	1	0.0031
H138	14	16	14	13	29	24	9	11	12	10	10	1	0.0031
H139	14	16	13	13	29	24	9	11	14	10	10	1	0.0031
H140	14	16	13	13	29	24	11	11	13	10	10	1	0.0031
H141	14	15	17	13	29	24	12	11	13	10	11	1	0.0031
H142	14	14	17	12	30	24	12	12	13	10	11	1	0.0031
H143	14	14	19	12	30	24	12	11	13	10	11	1	0.0031
H144	14	14	19	12	30	23	12	11	14	10	11	4	0.0125
H145	14	14	19	14	30	24	11	11	13	10	11	1	0.0031
H146	14	14	19	14	30	22	10	13	14	10	11	1	0.0031
H147	14	14	19	14	28	23	10	11	12	12	10	1	0.0031
H148	14	12	19	14	28	22	10	11	12	9	10	1	0.0031
H149	14	12	16	14	29	23	10	11	13	9	11	1	0.0031
H150	14	17	16	14	29	21	10	11	13	11	11	1	0.0031
H151	14	17	16	14	29	24	10	12	12	11	11	1	0.0031
H152	14	17	16	13	29	24	10	11	14	11	11	3	0.0094
H153	14	17	19	13	29	24	10	11	13	11	11	1	0.0031
H154	14	13	19	13	29	24	10	13	13	10	10	1	0.0031
H155	14	13	19	13	28	24	10	11	12	10	10	1	0.0031
H156	14	13	15	13	29	25	10	11	12	10	11	1	0.0031
H157	14	13	15	13	33	25	10	13	13	10	11	1	0.0031
H158	14	13	15	13	33	25	10	11	12	10	11	1	0.0031
H159	14	13	15	14	33	24	11	11	13	10	11	1	0.0031
H160	14	13	15	14	33	21	11	14	13	10	11	1	0.0031
H161	14	12	15	12	33	23	11	11	13	9	11	1	0.0031
H162	14	13	15	12	33	23	11	11	13	12	11	1	0.0031
H163	14	13	18	12	28	23	11	11	13	12	14	1	0.0031
H164	14	13	20	12	32	23	11	11	13	10	10	1	0.0031
H165	14	13	18	12	32	23	11	11	13	10	12	1	0.0031
H166	14	12	14	12	28	23	11	11	13	9	12	1	0.0031
H167	14	13	14	12	30	23	10	11	13	10	10	1	0.0031
H168	14	13	14		30	23	9	12	13	10	10	1	0.0031
H169	14	13	14	13	30	23	12	12	12	10	10	1	0.0031
H170	14	13	14	13	30	21	11	13	12	10	10	3	0.0094
H171	14	13	14	13	30	21	12	11	12	10	13	1	0.0031
H172	14	13	19	13	30	23	12	11	12	11	11	1	0.0031
H173	14	13	19	14	30	23	10	11	12	10	11	1	0.0031
H174	14	19	17	14	30	23	9	11	12	10	11	1	0.0031
H175	14	13	17	14	31	23	11	11	13	10	10	2	0.0063
H176	14	12	19	14	33	23	11	11	13	10	11	1	0.0031
H177	14	13	19	14	29	23	9	11	13	10	11	1	0.0031
H178	14	13	20	14	30	23	9	11	13	12	10	1	0.0031
H179	14	13	20	14	31	24	10	11	13	10	13	1	0.0031
H180	14	13	18	14	31	24	10	11	13	10	14	1	0.0031
H181	14	13	15	14	31	24	10	11	13	9	11	1	0.0031
H182	14	16	18	14	31	24	12	11	13	9	11	1	0.0031
H183	14	17	18	14	28	24	9	11	13	10	11	1	0.0031

Table 3. Contd.

H184	14	17	18	14	29	24	11	11	13	10	10	1	0.0031
H185	14	13	18	14	29	24	10	11	13	10	10	1	0.0031
H186	14	13	16	14	32	24	10	11	13	10	10	1	0.0031
H187	14	13	16	14	29	24	10	11	13	10	13	1	0.0031
H188	14	13	19	14	29	24	10	11	13	10	11	1	0.0031
H189	14	13	19	14	30	24	9	11	13	10	10	1	0.0031
H190	14	13	14	14	30	24	9	11	13	11	12	1	0.0031
H191	14	16	14	14	29	24	9	11	13	11	11	1	0.0031
H192	14	13	14	14	29	24	9	11	13	9	10	5	0.0156
H193	14	13	14	14	33	24	9	11	13	9	10	1	0.0031
H194	14	17	14	14	29	24	11	11	13	9	10	1	0.0031
H195	14	13	14	14	29	24	12	11	13	9	10	1	0.0031
H196	14	13	14	13	28	23	8	11	13	10	10	1	0.0031
H197	14	13	14	13	28	23	10	11	13	10	10	1	0.0031
H198	14	13	14	13	32	22	9	11	13	10	10	1	0.0031
H199	14	13	14	13	30	22	9	11	13	9	10	1	0.0031
H200	14	13	14	13	30	24	11	11	13	9	11	1	0.0031
H201	14	13	17	13	30	24	11	11	13	11	11	1	0.0031
H201	14	13	15	13	29	24	10	11	13	11	11	1	0.0031
H202	14	13	15	13	29	23	9	11	13	11	11	1	0.0031
H203	14	13	18	13	29	23	9	11	13	10	13	3	0.0094
H204	14	13	18	13	29	21	11	11	13	10	13	1	0.0031
H205	14	19	13	13	29	24	11	11	13	11	13	1	0.0031
H206	14	13	13	14	30	24	11	11	13	12	13	1	0.0031
H207	14	13	13	14	30	22	9	11	13	12	13	1	0.0031
H208	14	13	19	12	30	22	9	11	12	10	13	1	0.0031
H209	14	13	19	12	30	24	10	11	12	10	10	1	0.0031
H210	14	13	16	12	28	24	10	11	12	10	10	1	0.0031
H211	14	13	19	12	30	24	10	11	12	12	12	1	0.0031
H212	14	13	19	14	29	22	10	11	12	9	12	1	0.0031
H213	14	13	19	14	30	23	10	11	12	11	11	1	0.0031
H214	14	13	17	13	30	23	10	11	12	12	12	1	0.0031
H215	14	13	19	13	30	23	9	11	12	12	12	1	0.0031
H216	14	17	19	13	29	23	11	11	12	12	10	1	0.0031
H217	14	13	15	13	29	23	8	11	12	12	10	1	0.0031
H218	14	13	15	13	31	23	9	11	12	11	10	1	0.0031
H219	14	13	15	14	29	23	9	11	12	11	13	1	0.0031
H220	15	17	18	14	29	23	9	11	12	11	10	1	0.0031
H221	15	17	18	14	29	24	10	11	12	11	14	1	0.0031
H222	15	13	18	14	29	24	10	11	12	11	11	1	0.0031
H223	15	13	18	14	31	23	12	11	14	10	11	1	0.0031
H224	15	13	18	14	31	21	12	11	13	10	11	2	0.0063
H225	15	13	18	14	31	24	9	11	14	10	11	1	0.0031
H226	15	13	14	14	29	24	9	11	13	9	14	1	0.0031
H227	15	19	19	14	29	24	9	11	13	10	13	1	0.0031
H228	15	13	19	12	29	24	10	11	13	10	10	1	0.0031
H229	15	13	16	12	30	22	11	11	13	10	10	1	0.0031
H230	15	13	16	12	30	23	10	11	13	10	10	1	0.0031
H231	15	17	20	12	28	23	10	11	13	9	10	1	0.0031
H232	15	17	16	13	29	23	9	11	12	12	11	1	0.0031

Table 3. Contd.

H233	15	13	16	13	29	23	9	14	12	12	10	1	0.0031
H234	15	13	16	12	29	23	8	14	12	10	11	1	0.0031
H235	15	13	16	12	29	24	8	13	12	10	11	1	0.0031
H236	15	13	16	12	29	23	9	11	13	10	13	1	0.0031
H237	15	13	16	13	29	23	9	13	13	9	14	1	0.0031
H238	15	13	16	12	29	23	9	13	13	11	10	1	0.0031
H239	15	13	13	14	29	23	10	11	13	11	10	1	0.0031
H240	15	12	13	13	29	23	10	11	14	11	10	1	0.0031
H241	15	12	13	14	29	23	12	11	13	10	12	1	0.0031
H242	15	12	20	14	29	23	12	11	13	10	11	1	0.0031
H243	15	13	19	14	29	23	12	13	12	10	10	1	0.0031
H244	15	13	19	14	31	23	12	13	14	10	10	5	0.0156
H245	15	13	15	14	31	23	9	11	13	10	10	3	0.0031
H246	15	13	18	14	31	21	9	12	13	10	10	1	0.0031
H247	15	13	18	14	31	23	9	11	12	10	14	1	0.0031
H248	15	13	18	14	31	24	9	12	13	10	10	1	0.0031
H249	15	13	18	12	29	24	9	12	13	10	11	1	0.0031
H250	15	13	18	13	28	24	9	11	13	10	10	1	0.0031
H251	15	15	18	14	28	24	9	11	13	10	13	1	0.0031
H252	15	13	18	14	30	24	9	11	14	10	13	1	0.0031
H253	15	13	14	14	31	24	9	12	13	10	10	1	0.0031
H254	15	13	14	13	31	22	9	12	13	10	10	1	0.0031
H255	15	13	17	13	31	22	11	11	13	10	10	1	0.0031
H256	15	15	20	13	31	23	8	11	13	10	10	1	0.0031
H257	15	13	19	13	29	23	10	11	13	10	10	1	0.0031
H258	15	13	19	13	29	24	9	11	13	10	10	2	0.0063
H259	15	13	17	13	29	24	9	11	13	10	10	2	0.0063
H260	15	13	19	13	31	24	9	11	13	10	10	1	0.0031
H261	15	13	15	13	29	24	12	11	13	10	10	1	0.0031
H262	15	19	15	13	30	24	10	11	13	10	10	1	0.0031
H263	16	13	15	13	29	24	10	11	14	10	10	1	0.0031
H264	16	13	15	12	30	24	10	11	12	10	11	1	0.0031
H265	16	13	15	14	30	24	10	11	13	10	11	1	0.0031
H266	16	13	15	14	30	23	10	11	12	10	11	1	0.0031
H267	16	17	15	14	28	23	10	11	13	10	11	1	0.0031
H268	16	17	15	14	28	21	10	11	12	10	13	1	0.0031
H269	16	17	19	14	29	24	10	11	13	10	10	1	0.0031
H270	16	17	19	14	29	22	10	11	13	10	10	1	0.0031
H271	16	13	16	14	29	24	10	11	13	10	10	1	0.0031
H272	16	13	16	13	29	24	10	11	14	10	10	1	0.0031
H273	16	13	14	13	30	24	10	11	13	10	10	1	0.0031
H274	17	13	18	13	30	24	10	11	13	10	10	1	0.0031
H275	17	13	20	13	29	24	10	14	13	10	14	5	0.0156
H276	17	19	14	13	29	24	10	14	13	10	14	1	0.0031

N, Number of males observed for each haplotype; F, Frequency of each haplotype in the sample of 320 males.

between populations, compared with autosomal STRs. This can be attributed to the greater sensitivity of nonrecombining Y-chromosomal markers to founder effects and genetic drift. A similar conclusion was

Table 4. Comparison of the haplotypes and haplotype diversity in different human population groups.

Parameter	Iraq[1]	Tunisia[2]	Germany[3]	Italy[4]	China[5]	India[6]	India[7]
Individuals number	320	105	88	100	36	25	154
Haplotypes number	276	81	77	82	34	16	152
Unique Haplotypes	256	67	39	53	28	13	150
Proportion of unique haplotypes	0.93	0.83	0.51	0.65	0.82	0.81	0.98
Non-Unique Haplotypes	20	14	38	29	6	3	2
Proportion of non-unique haplotypes	0.07	0.17	0.49	0.35	0.18	0.19	0.01
Ratio (Unique : Non-Unique)	13.2	4.88	1.03	1.83	4.67	4.33	98
Haplotypes diversity	0.8392	0.9932	0.9963	0.9941	0.9968	0.950	0.9935

[1]This study. [2]Reference: Imen et al., 2005; [3]Reference: Manfred et al., 2001; [4]Reference: Manfred et al., 2001; [5]Reference: Manfred et al., 2001; [6]Reference: (Manfred et al., 2001); [7]Reference: (Kuppareddi et al., 2010).

reached recently by Forster et al. (2000), on the basis of a phylogenetic approach only. The use of Y STRs allows the simple construction of highly variable haplotypes (Henke et al., 2001; Hara et al., 2007; Imad et al., 2014a, b). With these haplotypes, it is possible to analyze differences in population structure by a comparison of haplotype diversity and of the number of population-specific haplotypes (Imad et al., 2014c, d).

Conclusions

Power of discrimination values for all tested loci means that those loci can be used as a DNA-based database. Different alleles were observed across the population. The lowest gene diversity was DYS392 and DYS439. The high gene diversity was DYS385b and DYS389II. Based on statistical parameters, the population of Iraq may use these 11 STR loci as a vital tool for forensic identification and paternity testing.

Conflict of Interests

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

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Full Length Research Paper

Micropropagation of *Jatropha curcas* superior genotypes and evaluation of clonal fidelity by target region amplification polymorphism (TRAP) molecular marker and flow cytometry

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The major limitation in large-scale cultivation of *Jatropha curcas* for use as energy crop is the inconsistent and unstable seed yield due to the heterozygous nature of the plant. A reliable *in vitro* regeneration system is necessary for continuous supply of quality planting material at large-scale. In this study, the interaction between the season collection of explants and capacity of *in vitro* regeneration shoots from foliar explants was investigated. Three genotypes selected in our breeding program were evaluated. We achieved an average of 39.8, 25.5 and 10.9 shoots per explant for G1, G2 and G3 genotypes, respectively. All genotypes showed higher regeneration capacity when the foliar explants were collected in September/2012 season. Excellent results were obtained with the use of micrografting technique for the *in vitro* rooting, with a plant recovery rate of 85%. In order to confirm the genetic stability of micropropagated shoots, two analyses were performed: ploidy estimation using flow cytometry and DNA polymorphism analysis using TRAP molecular markers, which has been here reported for the first time for *J. curcas*. For G1 genotype, it was found that 4% of the plants were tetraploid and 5% of plants had polymorphic bands. No DNA polymorphisms were found in plants of other genotypes. Thus, the low or no somaclonal variation indicates that the protocol established preserves the clonal fidelity of micropropagated plants.

Key words: Organogenesis, *in vitro* micrografting, foliar explants.

INTRODUCTION

Jatropha curcas L. (Euphorbiaceae) is a perennial deciduous and monoecious shrub (3 to 10 m), native to México and Central America (Achten et al., 2008). Recently, the species has attracted the attention of the

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Abbreviations: BAP, 6-Benzylaminopurine; **GA_3,** gibberellic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **TDZ,** Thidiazuron; **TRAP,** target region amplification polymorphism.

international research community due its potential for production of exceptional quality biodiesel from the oil contained in its seeds, more so in view of the potential for avoiding the dilemma of "food x fuel" (Ghosh et al., 2007; Carels, 2011). *J. curcas* oil yield per hectare is around 2000 L which can be increasing significantly by selective breeding. Its seed contain 25 to 40% oil with predominance of oleic fatty acid in triglycerides which can collaborate with oxidative stability to biodiesel (Argollo Marques et al., 2013). Moreover, the plant is considered as drought tolerant, low seed cost, has wide adaptation to different soil and climate conditions and ability of biodiesel to persist stable upon storage (Balat, 2011; Gomes et al., 2010). Despite its potentiality, the oilseed is a non-domesticated plant and still there are none cultivar for commercial plantations. The specie is xenogamic and highly heterozygous for most agronomic traits suggesting a high degree of segregation (Argollo Marques et al., 2013). Consequently, breeding programs require several years to obtain a stable cultivar.

Biotechnological tools such as *in vitro* cloning of superior genotypes may contribute to speed up the breeding process meeting the growing demand for stable and improved genetic material in the near future (Argollo Marques et al., 2013). Numerous studies have been reported on *J. curcas* organogenesis (Sujatha and Mukta, 1996; Lu et al., 2003; Wei et al., 2004; Sujatha et al., 2005; Sharma et al., 2006; 2011; Rajore and Batra, 2007; Deodore and Johnson, 2008; Kumar and Reddy, 2010; 2012; Kumar et al., 2010a; b; 2011a; Singh et al., 2010; Khurana-Kaul et al., 2010) and somatic embryogenesis (Sardana et al., 2000; Jha et al., 2007; Cai et al., 2011). Though several reports on regeneration from various explants in *J. curcas* exist, none report on the influence of season collection of explants and its interaction with different genotypes are available. Besides the genotype, the physiological, nutritional and sanitary condition of the mother plant strongly influences the *in vitro* regeneration process. Also, important is the choice of suitable culture medium and growth regulators combinations for shoots induction/elongation and *in vitro* rooting, the most critical phase. Although, previous studies have reported relative success on *in vitro* rooting of micropropagated plants, the rooting rates have been low and not exceeded 51.1% (Kumar and Reddy, 2010). Alternatively, this difficulty can be overcome using *in vitro* micrografting techniques (Silva et al., 2005). Other challenge is the generating planting material with genetic stability in order to maintain interest traits of superior genotypes (Rahman and Rajora, 2001). The somaclonal variations are genetic and epigenetic changes that occur uncontrollable, spontaneous and randomly during the *in vitro* process. It is a phenomenon that is highly undesirable of obtaining genetically uniform clones. The level of somaclonal variation may be influenced by choice of the regeneration method, genotypes, explants origin, kind and concentration of growth regulation and number and duration of the subcultives (Ahuja, 1987; Rani and Raina, 2000; Bairu et al, 2011).

Morphological, physiological/biochemistry, cytogenetic and molecular techniques can be used to detect somaclonal variations (Kwon et al., 2010; Song et al., 2012; Suman et al., 2012). Flow cytometry (Kaewpoo and Te-Chato, 2010) and RAPD and AFLP molecular markers (Sharma et al., 2011; Leela et al., 2011) were recently used to assess the chromosomal and sequence changes, respectively and assess the genetic fidelity of micropropagated plants of *J. curcas*. Target region amplification polymorphism (TRAP) is a kind of developed molecular marker system with the advantages of simplicity, high throughput, numerous dominant makers and highly reproducibility. Another advantage of TRAP in relation to other markers would be use as parts of known sequences of genes of interest as a fixed primer and a random primer that amplifies the adjacent regions of this gene (Hu and Vick, 2003). TRAP molecular marker may be useful in genotyping of germplasm banks and tagging genes of agronomic traits of interest in the cultures, the large amount of information that is generated (Hu and Vick, 2003). This technique can also be applied in order to assess changes at the DNA level in micropropagated plants to confirm the genetic stability and clonal fidelity. In accordance with Sharma et al. (2011), this kind of study should be carried at the first stages of culture in order to eliminate the possible variants and avoid its micropropagation.

This paper reports efficient micropropagation protocols via organogenesis using foliar explants for the cloning of three *J. curcas* genotypes selected in our breeding program due to its superior oil yield and quality. We investigated the interaction between the genotypes studies and the season collection of explants. The micropropagated plants were successfully rooting by use of micrografting technique. Moreover we have established for the first time, a methodology to assess the somaclonal variation in regenerated plants using polymorphic TRAP molecular markers. Additionally, we also used flow cytometry to detect changes at the level of chromosomes.

MATERIALS AND METHODS

Plant material

Matrix plants used for this study were cultivated at the experimental field, (not irrigated) situated in Campinas/São Paulo, Brazil (latitude 22°53' S; longitude 47°5' W and altitude 664 m). Three genotypes of *J. curcas* (G1, G2 and G3) were selected by our breeding program based on superior agronomic characteristics such as yield and oil content and quality.

Foliar explants organogenesis

Young apical fully developed leaves were collected from G1, G2 and G3 plants and were stored in the dark at room temperature for

Table 1. Description of the growth regulator treatments used for *in vitro* organogenesis of physic nut explants.

S/N	Description
1	Control - no growth regulators
2	1.5 mg L^{-1} TDZ
3	3.0 mg L^{-1} TDZ
4	0.2 mg L^{-1} IBA/0.5 mg L^{-1} TDZ
5	0.2 mg L^{-1} IBA/1.0 mg L^{-1} TDZ
6	0.2 mg L^{-1} IBA/2.0 mg L^{-1} TDZ
7	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/0.5 mg L^{-1} TDZ
8	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/1.0 mg L^{-1} TDZ
9	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/2.0 mg L^{-1} TDZ

20 h. The sterilization process involved immersion in sodium hypochlorite solution (2.5% v/v) for 15 min, followed by rinsing three times in sterile distilled water. The leaves were cut into 0.7 x 0.7 cm pieces and cultured in MS (Murashige and Skoog, 1962) medium, supplemented with 100 mg L^{-1} inositol, 10 mg L^{-1} cysteine, 25 mg L^{-1} reduced glutathione, 30 g L^{-1} sucrose, 500 mg L^{-1} hydrolyzed casein, 6 mg L^{-1} copper sulfate and different combinations and concentrations of plant growth regulators (Table 1). The culture medium was solidified using 2.4 g L^{-1} phytagel, and the pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 120°C and 1.2 atm for 20 min. The explants were maintained in culture medium under a 16 h photoperiod at 25 ± 1°C, with subculture every 21 days. Each treatment comprised five repetitions, and one repetition was represented by the average of three explants. The experiments were repeated during three different periods: May 2011, November 2011 and September 2012. The number of shoots per explant was recorded after six weeks of culture.

Elongation of regenerated shoots

Regenerated shoots of the G1 and G2 genotypes (5 mm in length) were incubated in MS medium supplemented with 10 mg L^{-1} cysteine, 25 mg L^{-1} reduced glutathione, 500 mg L^{-1} hydrolyzed casein, 6 mg L^{-1} copper sulfate, 50 mg L^{-1} adenine sulfate, 30 g L^{-1} sucrose, 7 g L^{-1} agar and different plant growth regulators. Different concentrations of gibberellic acid (GA_3) (1.5, 3.0 mg L^{-1} and 4.5 mg L^{-1}) were tested in the first experiment, and three combinations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (0.15 /0.05, 0.3 /0.1 and 0.5 /0.2 mg L^{-1}) were tested in a second experiment. Each treatment was included ten repetitions, with each replicate consisting of three shoots on average. The explants and shoots were maintained in culture medium under a 16 h photoperiod at 25 ± 1°C, with subculture every 21 days. The number of leaves and length of the shoots were recorded after six weeks of culture.

In vitro micrografting and acclimatization

Shoot apices of 5 mm in length were isolated from the regenerated plants and used for *in vitro* micrografting onto *J. curcas* L. seedlings. Seeds without tegument were surface sterilized in sodium hypochlorite (2.5% v/v) for 20 min, rinsed three times in sterile distilled water and inoculated in solid MS medium containing 30 g L^{-1} sucrose and maintained under a 16 h photoperiod at 25 ±1°C for 40 days to obtain *in vitro* rootstocks. *In vitro* micrografted plants were incubated in MS medium supplemented with 30 g L^{-1} sucrose, 0.15 mg L^{-1} BAP and 0.05 mg L^{-1} IBA. The number of developed graft plants, the number of leaves and the leaf lengths were recorded at 30 days after inoculation. The experiment was repeated twice with ten replicates each and one plant per tube. All micrografted plants developed *in vitro* were transferred to flasks containing sterile vermiculite for acclimatization. The plants were maintained under high humidity for 40 days, followed by the gradual reduction of the humidity. The plants were transferred to pots containing a mixture of soil and commercial substratum (Plantimax ™/Eucatex) in a 1:1 ratio and maintained under greenhouse conditions.

Statistical analysis

All the experiments were set up in factorial completely randomized design. Statistical analyzes were performed using the SANEST program (Machado and Zonta, 1995). Data from the *in vitro* organogenesis and shoot elongation experiments were subjected to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) to determine the significance of the differences among the means. The organogenesis data were transformed by $\sqrt{x+0.5}$ before analysis.

Characterization of *in vitro*-regenerated shoots

Two groups of *in vitro*-regenerated shoots that had been subcultured in MS medium supplemented with 0.3 mg L^{-1} BAP /0.1 mg L^{-1} IBA were used for genetic stability analyses using flow cytometry and TRAP molecular markers.

Flow cytometry

The first group was analyzed on 2º subculture (20 shoots of each genotype: G1 and G2) and 12º subculture (30 shoots of genotype G1), and the second group was analyzed on 7º subculture (10 shoots of each genotype: G1, G2 and G3) using flow cytometry. The ploidy level of the regenerated shoots was determined by flow cytometry using the CyFlow Ploidy Analyzer (Partec Gmbh.) equipped with an UV-LED lamp. Nuclear suspensions were isolated from pieces of shoot leaves with area of 0.25 cm^2. The cell nuclei were exposed using a steel scalpel blade and stained using the CyStain UV ploidy solution kit (Partec Gmbh.), which uses 4-6-diamidino-2-phenylindole (DAPI) as a fluorochrome. The nuclei solution was filtered through a 30 µm filter and immediately analyzed. One thousand intact nuclei were evaluated in each sample. The histograms were analyzed using CyView software (Partec Gmbh.). The samples with coefficients of variation (CV) greater than 10% were discarded. The ploidy level of each sample was compared with that of a leaf from the donor plant.

Molecular marker analysis

Genomic DNA from the shoots of G1 (20 shoots), G2 (10 shoots) and G3 (10 shoots) genotypes was extracted from the leaves using the method of Doyle and Doyle (1990). The DNA polymorphisms in the samples were assessed using TRAP molecular markers. The PCR reactions were performed in final volume of 13 µL containing 1X PCR buffer, 2.7 mM $MgCl_2$, 115 µM of each dNTP, 0.6 µM of fixed and arbitrary primers, 0.6 U Taq DNA polymerase and 100 ng of genomic DNA. The PCR reaction was initiated at 94°C for 2 min, followed by 5 cycles of 94°C for 45 s, 35°C for 45 s and 72°C for 1 min; 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 1 min; and a final cycle of 72°C for 7 min. Twelve different combinations of primer pairs were used. The fixed *primers* were designed according to Cristofani-Yali et al. (2007) for the citrus genes, and the arbitrary

Table 2. Description of the fixed and arbitrary *primers* used in the TRAPs molecular marker technique.

Identification	Gene	Type	5' → 3'
01F	*ACC synthase*	Fixed	TCCCCGAGGCACAGCATC
02F	*Caffeic acid O-methyltransferase*	Fixed	ACAGGGCCAAAGGTAAAC
05F	*NADP-dependent glyceraldehyde 3-phosphate dehydrogenase*	Fixed	ACGCGTCCGCCACTCTCA
06F	*Chlorophyll a/b- binding protein*	Fixed	TGGCAGCATCGTCAACT
07F	*SRG1*	Fixed	GGCACCGCACTCACCATC
08F	*Miraculin-like protein 2*	Fixed	GTGGCGAATTTTGACTGT
10F	*Ein3-like protein*	Fixed	CAGTTTCTTGTTGCTACG
02R	*Caffeic acid O-methyltransferase*	Fixed	AGCGCGTCCTGGTGATGC
03R	*Sucrose synthase*	Fixed	ATATACCCCAGCCAATGT
06R	*Chlorophyll a/b- binding protein*	Fixed	GGAGACGGCGGGCTTAGA
07R	*SRG1*	Fixed	TGCTCTGGTTTCGGACAA
09R	*DNAJ*	Fixed	CGCATCCTCGCCGTATTG
P2	-	Arbitrary	GACTGCGTACGAATTTGC
P4	-	Arbitrary	GACTGCGTACGAATTTGA

primers were designed according to Li and Quiros (2001) (Table 2). Formamide was added at an equal volume to the amplified PCR products for the posterior denaturation step, involving heating at 94°C for 3 min. Electrophoresis was performed using a 5% denaturing polyacrylamide gel containing 7 M urea and TBE buffer under the following conditions: fixed power (75 W) for 2 h, followed by gel staining using silver nitrate (0.2%), according to Creste et al. (2001). DNA polymorphisms were identified by comparing the profiles of the PCR-amplified fragments of the regenerated shoots with those of the respective donor plants.

RESULTS AND DISCUSSION

Foliar explants organogenesis

The season collection of foliar explants into matrix plants had a great influence in the shoot regeneration of the three studied genotypes. All genotypes had better responses when the leaf explants were collected in September 2012 (Figure 1). The G1 genotype showed great oscillations in the regeneration capacity with averages of 10.2, 1.4 and 24.8 shoots per explant in May 2011, November 2011 and September 2012, respectively. The other two genotypes had smaller oscillations during the periods analyzed (Figure 1). September represents the beginning of the spring season in the southern hemisphere, a time with warmer days and the beginning of the rainy season. This climate induces the growth of new branches and leaves by physic nut plants, a deciduous species. Despite all explants were collected from fully developed leaves, those that were collected during this period (September/2012) were likely younger than those that were collected during May and November, 2011. Therefore, it is expected that the climate and explant age positively influenced the induction of *J. curcas* organogenesis during the experiment realized in September 2012.

In two season collections (May/2011 and September/2012) the results demonstrated the occurrence of genotype dependence in the *J. curcas* organogenesis using foliar explants. Similar results were found by other authors who reported different responses in the direct organogenesis induction of various *J. curcas* genotypes using cotyledonary explants (Kumar et al., 2010b) or petiole explants (Kumar and Reddy, 2010). These studies considered that the different responses of genotypes to organogenesis may be related to different levels of endogenous hormones (especially cytokinin) found in each genotype. The effect of different culture media in each genotype was only performed in experiment realized during September 2012, in which regeneration rate was superior. In this season collection of explants were achieved an average of 39.8, 28.9 and 10.9 shoots per explants for the genotypes G1, G2 and G3, respectively (Figure 2). These averages were higher than those reported by other authors (3.5 to 10 shoots per explants) (Shukla et al., 2013; Zhang et al., 2013). For all genotypes, the control treatments without growth regulators (treatment 1) did not regenerated none adventitious shoot. For G1 and G3 genotypes, the combinations of thidiazuron (TDZ) and IBA (treatments 4 to 9) were more efficient than the treatments containing TDZ alone (treatments 2 and 3). Different results were observed for the G2 genotype, for which treatments with TDZ alone also effectively induced the regeneration of shoots.

Our results show that the combinations of TDZ and IBA were the most efficient in *J. curcas* shoot regeneration from foliar explants (Figures 2 and 3a). Notably, BAP did not affect the rate of shoot regeneration, therefore, the use of this hormone was considered unnecessary. Similar results were verified by Khurana-Kaul et al. (2010) who also showed that the combination of TDZ and

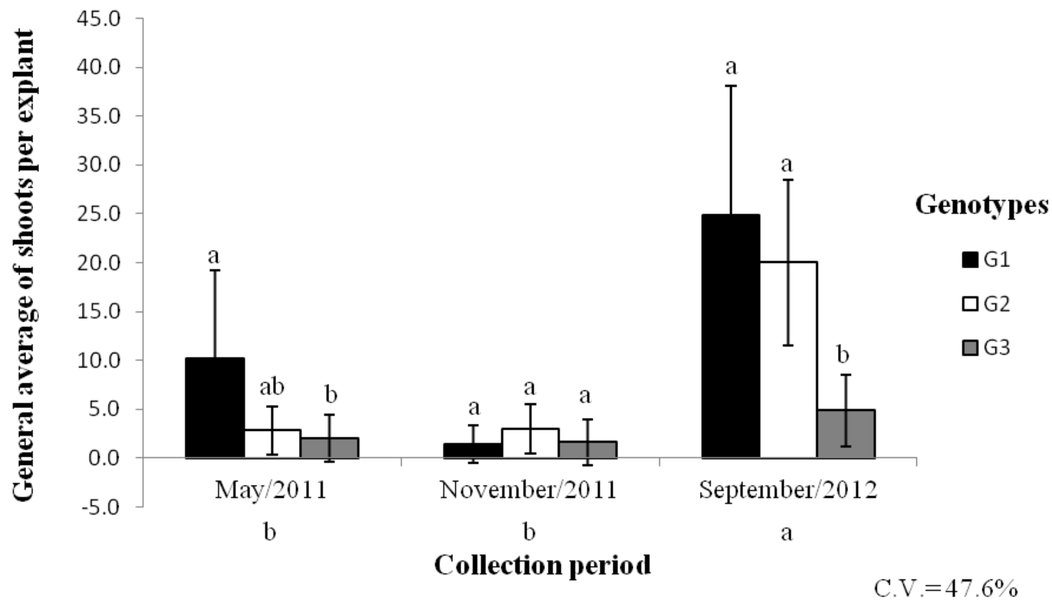

Figure 1. Mean of number of regenerated shoots per explant from somatic organogenesis of physic nut. Data are represented by the average of all treatments (medium) used for the physic nut genotypes during different season collection. The mean values with different letters between genotypes in the same period and different letters between collection period differ according to Tukey's test (p<0.05).

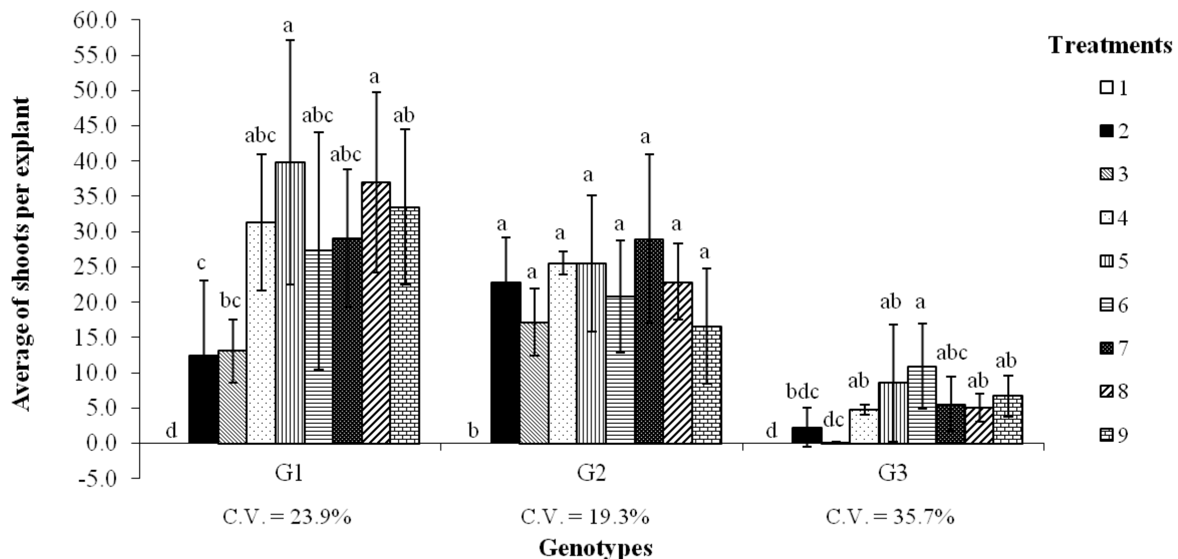

Figure 2. The mean of number of regenerated shoots per explant in nine treatments (medium) used to induce the somatic organogenesis of physic nut explants of different genotypes. Data for September 2012 experiment are shown. The mean values with different letters for the same genotype differ according to Tukey's test (p<0.05).

IBA was more effective than the combination of BAP and IBA in *J. curcas* shoot regeneration using foliar segments as explants.

Elongation of the shoots

High percentage of shoot oxidation was observed in both genotypes evaluated (G1 and G2) in the presence of GA_3 in the culture media. Despite oxidation process, treatment containing 4.5 mg L^{-1} GA_3 promoted a good shoots elongated rate (10.3%) and the best average length (0.97 cm) for G1 shoots. For G2 genotype the higher elongated shoots rate (16.6%) was obtained in treatment without GA_3, with an average length of 0.50 cm. The combination of BAP (0.3 mg L^{-1}) and IBA (0.1 mg L^{-1}) promoted the

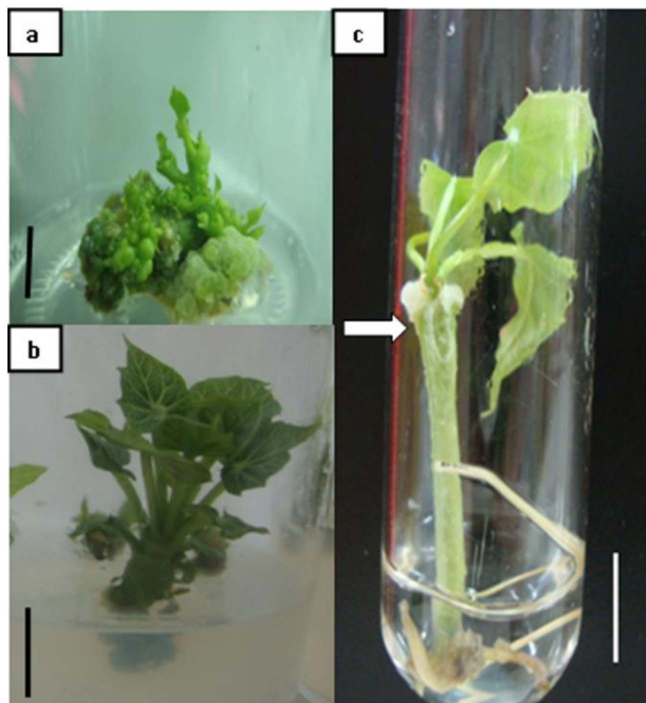

Figure 3. a) Shoot formation through somatic organogenesis from genotype G1 leaf explants. **b)** Shoot elongation from genotype G1 in culture medium containing BAP and IBA. **c)** Micrografted physic nut plant. Bar = 1 cm.

Table 3. Physic nut shoots elongation for genotypes G1 and G2 using different concentrations of BAP and IBA.

Treatment	Shoot Size (cm)		Number of Leaves	
	G1	G2	G1	G2
B1 (Control)	0.6^{a}	0.6^{a}	3.1^{a}	2.3^{ab}
B2 (0.15 mg L^{-1} BAP + 0.05 mg L^{-1} IBA)	0.9^{a}	0.7^{a}	3.8^{a}	1.6^{b}
B3 (0.3 mg L^{-1} BAP + 0.1 mg L^{-1} IBA)	1.4^{a}	1.0^{a}	4.5^{a}	3.5^{a}
B4 (0.5 mg L^{-1} BAP + 0.2 mg L^{-1} IBA)	0.6^{a}	0.7^{a}	2.5^{a}	3.6^{a}
C.V.	31.2%	31.7%	28.5%	35.1%

Average values with different letters in each column differ according to Tukey's test ($p<0.05$).

best shoot elongation (1.4 and 1.0 cm) and the largest number of leaves per shoot (4.5 and 3.5) for G1 and G2 genotypes, respectively (Table 3 and Figure 3b). The increasing of plant growth regulator concentration was directly proportional to increasing of percentage of elongated shoots for G1, ranging from 30% (control, without plant growth regulators) to 68.2% (B4 treatment: 0.5 mg L^{-1} BAP/0.2 mg L^{-1} IBA). The B4 treatment also reduced explant oxidation from 65 (control) to 9.1%. Approximately, 59.1 and 25% of the shoots cultivated in the B4 treatment formed basal calli in G1 and G2 explants, respectively. Basal callus formation is not desirable because this can hamper the posterior stage of *in vitro* rooting, the most critical of organogenesis process in *J. curcas*. Therefore, it was possible to conclude that the use of BAP and IBA in the culture medium was more efficient than GA_3 for shoot elongation.

In vitro micrografting of shoots and acclimatization

The *in vitro* micrografting of *J.curcas* shoots onto seedlings of the same species was successful (Figure 3c). We obtained 85% of developing shoots of 20 *in vitro* micrografted plants. The mean number of leaves and the mean leaf length of the micrografted plants were 2.1 and 1.1 cm, respectively, after 30 days of culture. The plants also showed numerous well-developed roots. There are many reports of lower *in vitro* rooting percentages (22 to 52%) for *J. curcas* micropropagated shoots (Kumar et al.,

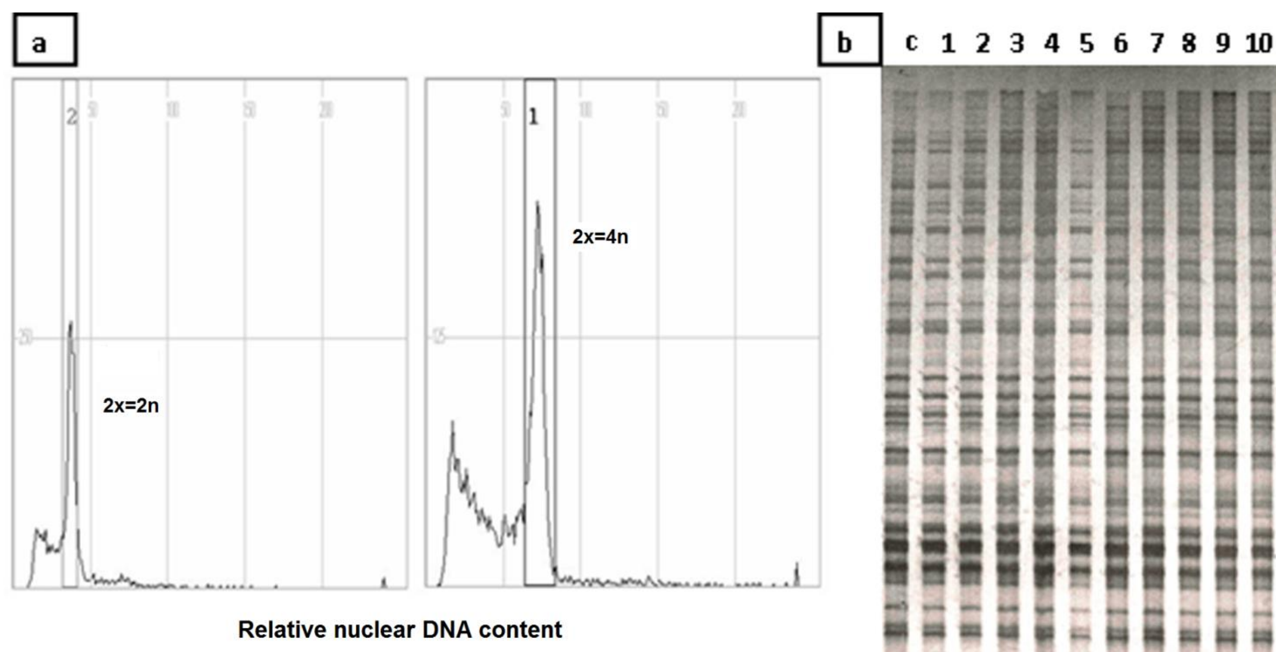

Figure 4. a) Histogram obtained from the results of the flow cytometry analysis of two *in vitro*-regenerated physic nut plants: one diploid and the other tetraploid. **b)** Electrophoretic analysis of DNA amplification through TRAPs molecular markers showing genetic similarities among donor plants **(c)** and *in vitro* shoots (1-10).

2010b, 2011b; Kumar and Reddy, 2010), with rare exceptions, such as 83% obtained by Daud et al. (2013) using woody plant medium containing IBA and phloroglucinol and 86% obtained by Li et al. (2008) using MS medium supplemented with IBA. This difficulty has also been observed in other woody plants. Silva et al. (2005) compared the efficiency between two techniques, shoot rooting and *in vitro* micrografting, for the development and recovery of *in vitro* shoots of the 'Pera' sweet orange. The results showed 58% of plants rooted when the *in vitro* rooting was used and 100% of the plants recovered after *in vitro* micrografting. The high efficiency of the plants recovery in this study showed that *in vitro* micrografting might be a viable alternative as a replacement for the induction of *in vitro* rooting in shoots micropropagated via organogenesis. This is the first report in *J. curcas* micrografting. In addition, the acclimatization of micrografted plants was also considered successful because an average of 76.5% of plants survived. These plants might be transferred to the greenhouse after 30 days of acclimatization.

Ploidy of the regenerated shoots

Flow cytometry analysis of G1 shoots belonging to first group (second subculture) showed that 96% of the regenerated shoots were diploid and 4% were tetraploid (Figure 4a). The analysis performed in the same genotype after the 12th subculture showed the same proportion of tetraploidy plants. We can suggest that the variation in the ploidy level occurred at the beginning of *in vitro* culture and it was perpetuated during ten *in vitro* subcultures of shoots in the elongation medium. The regenerated shoots of the G2 genotype showed no changes in the ploidy level, yielding 100% diploid shoots after two subcultures. The same result was observed in the second group of plants in which 100% of the shoots evaluated after the 7th subculture (G1, G2 and G3 genotypes) were diploid, with ploidy levels identical to those of the donor plants. Kaewpoo and Te-Chato (2010) obtained similar results using epicotyl and hypocotyl *J. curcas* explants for callus and shoot induction. The callus, leaves and stem shoots were analyzed by flow cytometry, showing no variation in ploidy.

DNA polymorphisms in regenerated shoots

In accordance with established methodology in this paper, TRAP molecular markers resulted in a total of 266 bands, with an average of 22.2 bands per *primer* pair. In comparison with the donor plant, four polymorphic fragments were observed (1.5% of the total) in the molecular profile of genotype G1. All polymorphic fragments were in the same shoot sample. This polymorphic sample represented 5% of the total samples evaluated for this genotype. No polymorphism was

detected in the micropropagated shoots of the G2 and G3 genotypes (Figure 4b). The low DNA polymorphism observed in G1 genotype may be related to the high concentration of plant growth regulators used at the beginning of *in vitro* culture process, mainly in the shoot induction stage.

Sharma et al. (2011) observed similar results after evaluating the meristematic explants (axillary shoot buds) of *J. curcas* using others molecular markers (RAPD and AFLP). The authors also related few or no polymorphism among the genotypes studied. Leela et al. (2011) using RAPD analysis also recorded no somaclonal variation of regenerants. In accordance with some authors, the use of pre-existing meristems such as shoot tips and axillary buds from the hardwood shoot cuttings lower the risk of somaclonal variations (Ahuja, 1987; Ostry et al., 1994; Wang and Charles, 1991). These kinds of competent and pre-determined explants have less necessity of high growth regulator concentrations for micropropagation process when compared with organogenesis or somatic embryogenesis process using no meristematic explants. Others factors like type and concentration of plant growth regulators used in the medium, rate of multiplication, formation of adventitious shoots, increased culture period and genotype influence the rate of somaclonal variation (Bairu et al, 2011; Sharma et al., 2011). Our results show that even using non meristematic explants (leaf segments) and indirect regeneration *in vitro* (with prior callus formation) process has been possible to establish efficient and specific protocols for *in vitro* cloning of superior genotypes selected in our breeding program. Besides highly efficient on the shoots induction, these established protocols showed genetic stability of the micropropagules obtained.

Additionally, TRAP molecular markers identified polymorphism among the donor genotypes in 7.1% of the bands. 100% of DNA polymorphism was observed among genotypes with different origins. It is observed that G1 and G2 genotypes, originating from Brazil, showed identical molecular profiles, but both genotypes differed from G3, originating from Mexico. These results indicated the possibility to use these molecular markers for study of genetic diversity in *J. curcas* germplasm.

Kwon et al. (2010) evaluated the genetic diversity of *Vicia faba* L. germplasm and verified that TRAP molecular markers were able to efficiently distinguish divergent groups from different geographical origins. Creste et al. (2010) used TRAP molecular markers with primers for the genes likely involved in sucrose metabolism and the drought response to establish different clusters for 60 sugarcane genotypes. At the same time, similar studies has been realized by our time using fixed primers designed from specific ESTs involved in oil and phorbol esters metabolisms. The results (yet not published) will be helpful for the study of the genetic variability of these traits in our germplasm collection.

The results obtained in this work suggest low somaclonal variation in G1 elite genotype which was found only 4% of tetraploid plants and 1.5% of polimorfic bands. Zero somaclonal variation was founded in G2 and G3 superior genotypes. These variations were detected in beginning stage of organogenesis process. Thus, the results indicate adequate genetic stability and clonal fidelity of micropropageted superior genotypes by protocols now established. The superior traits conservation is very important for commercial production of *J. curcas* clonal variety. In addition, the *in vitro* cloning protocols established can be used to introduce genes of interest into the superior genotypes via genetic engineering as our team have done currently (data yet not published).

Conclusion

The season collection of foliar explants into matrix plants had a great influence in the shoot regeneration of the three studied genotypes. All genotypes had better responses when the leaf explants were collected in September 2012. There was genotype dependence in the organogenesis process: the G1 genotype showed a better shoot regeneration average than the other two genotypes independently of explants season collection. Micrografting proved to be a promising technique as a substitute during *in vitro* rooting of regenerated shoots, with a plant recovery rate of 85%. The evaluation of genetic homogeneity in tissue culture regenerates of *J. curcas* using Flow Cytometer and TRAP molecular marker showed low or no somaclonal variation indicating that the protocol established preserves the clonal fidelity of micropropagated plants.

Conflict of Interests

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

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Full Length Research Paper

Abnormal mitosis in root meristem cells of *Allium cepa* L. induced by a fabric dye reactive turquoise blue (Procion MX)

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Reactive Turquoise Blue (Procion MX) is a fabric dye used in small scale cotton fabric industries in various parts of India. The impact of this chemical on human health in the surrounding areas that discharge effluents is of serious concern. This needs to be assessed for short and long term effect on human genome. This investigation was aimed to find mitotic abnormalities as cytological evidence induced by the dye in root tip cells of onion (*Allium cepa* L.) grown in different concentrations: 0.01, 0.05, 0.1, 0.5 and 1.0% (weight per volume) prepared in distilled water in separate treatment schedules for 24 and 48 h. Mitotic aberrations (MA) were scored after staining with 2% acetocarmine by conventional squashing method. Root growth at various concentrations and duration of exposure of the dye were analyzed as macroscopic parameter for testing the cytotoxicity. Percentages of mitotic cells were analyzed as microscopic parameter to find the trend of mitotic indices and depression. Total abnormality of cells in percentage indicated the genotoxic assault of the dye. At higher concentrations, the root tip cells died in 24 h. Highest number of dividing cells with largest mitotic index value was observed at 0.01% exposed for 48 h. The abnormalities of common occurrences observed were unequal cytokinesis/ karyokinesis, formation of micronuclei, bi-nucleated cells and little condensed chromosomal arms in abnormal metaphase, anaphase and telophase. The abnormal mitotic cells were assumed to be due to genotoxic assault of the dye on chromosomal condensation mechanism resulting in very unusual long arms in rapidly dividing meristematic cells.

Key words: Reactive torquoise blue, genotoxicity, *Allium cepa*, mitotic aberration.

INTRODUCTION

Clothes and fabrics form the basis of human aesthetic senses and beauty and are given utmost importance from the beginning of civilization. To make them colourful, attractive and lucrative to customer for better commercialization, an array of various dyes are employed throughout the globe. Dyes are widely used in various industries like textile, rubber,paper, printing, colour photography, pharmaceuticals, cosmetics and

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many more (Raffi et al., 1997) and are normally manufactured from the primary products obtained through the distillation of coal-tar (Tyagi and Yadav, 2001). The textile industry utilizes mostly reactive dyes, which are used in dyeing cellulose fibres and accounts for the largest consumption of dyestuffs, at nearly 80% (Mathur et al., 2005a). India is the second largest exporter of dyestuffs after China (Mathur et al., 2005b). The annual global production of dyes and pigments is more than 0.7 Mt of which 10 to 15% is discharged into the environment without any proper treatment (Vinu and Madras, 2009).

Textile effluents are some of the most troublesome wastewaters to treat effectively because of their low biodegradability, significant toxicity and varied composition (Riga et al., 2005). The synthetic origin and complex aromatic structures of the dyes make them stable and difficult to be biodegraded (Seshadri et al., 1994; Fewson, 1998). Excessive use of these chemicals and ready disposal of their wash-off effluents to nearby agro-fields as well as drinking water resources poses problems on the surrounding biomass. It contaminates soil surface and ground water as well as food chains affecting health of the inhabitants of aquatic and terrestrial environment.

Textile dyes are mixtures of chemicals which contain salts, calcium stearate, carboxymethyl cellulose (CMC) and other unknown chemicals. The textile industry utilises mostly reactive dyes, which are used in dyeing cellulose fibres like cotton which accounts for about 40% of world fibre production (http://www.utexas.edu/centers/natstat/data/pdf). Fibre reactive dyes attach permanently to cellulose fibers using a covalent bond. These molecules carry a chromophore which absorbs various spectrum of light, allowing only certain spectrum to reflect. During the dyeing processes about 10 to 90% of the dye do not bind to the fibers and therefore are released into the environment (Zollinger, 1991; Abadulla et al., 2000). The most obvious impact of the discharge of dye coloured effluent is the persisting nature of the colour. It is stable and fast, difficult to degrade, toxic, rendering the water unfit for its intended use. Such dyestuffs can reach the aquatic environment, primarily dissolved or suspended in water, the conventional treatment of wastewaters from textile mills and dyestuff factories are unable to remove most of the azo and other dyes effectively. The resulting dye effluents may contain some components or moieties that could be toxic, carcinogenic or mutagenic to aquatic life (Suzuki et al., 2001).

Procion is a brand of fibre reactive dyes and Procion MX are a class of cold reactive dyes including Reactive Turquoise Blue which is a widely used cotton linen dyeing chemical in many parts of the world and are extensively used in tie dye and textile industry. It is widely used in cotton linen dyeing industries throughout Australia, Europe and US (http://en.wikipedia.org/wiki/ Reactive_ dye). It has cyclic structure with two chlorine atoms on it and these are the reactive sites that react with OH groups on cellulose fiber to create the strong covalent bonds responsible for the dichlorotazine giving extremely high fastness. The dye in commercial form represents a mixture of different chemicals. It is therefore not possible to know the exact concentration of the dye itself in the powder form of the commercial dyestuff and there could be other components in this mixture that could also be toxic. There may be one or two sulfonate groups on the phthalocyanine ring, two or three dichlorotriazine sections per copper phthalocyanine and the positions of these different items on the phthalocyanine ring are unknown and presumably random. In addition, this dye molecule, especially if kept in solution for several days, tends to 'stack up' to form aggregates of two or more molecules, causing the hue to shift to be more blue and less green. In tie and dye industries, the resulting textile wastewater is of a deep blue colour that affects water quality by inhibiting the penetration of sunlight and thus reducing photosynthetic activity (Lambrecht et al., 2007).

Human being is exposed to low levels of such toxic substances by direct or indirect way via irrigation, drinking, bathing, pisciculture and consuming many aquatic food species throughout his/her life time. The health hazard posed by such dyes is a major concern all the time. The immediate detrimental effects of these hazardous chemicals on human health are apparent while other long range effects are only dimly perceived. Concern for genotoxicity caused by environmental pollutants has led to the development of several biological tests for detecting and identifying genotoxicants in air, water and soil. According to Wollin and Gorlitz (2004), several azo dyes have shown genotoxicity by studying human keratinocytes (HaCaT cells).

Over the past decade, issues of animal use and care in toxicology research and testing have become one of the fundamental concerns for both science and ethics. Emphasis has been given to use of alternatives to mammals in testing, research and education (Mukhopadhyay et al., 2004). Organisms used in mutagenesis testing should be selected using criteria that permit a realistic evaluation of the potential of a suspected mutagen to induce changes in genetic material such as structural and/or numerical modification of chromosomes resulting in chromosome aberrations (Matsumoto et al., 2006).

Plants are direct recipients and are important model for genetic tests and environmental monitoring and use of plants for evaluation of environmental pollutants was validated (Cabrera et al., 1994). According to Vieira and Vicentini (1997) the effect of mutagens on eukaryotic nuclei can be assessed cytologically by observing inhibition of cell growth or division, interruption of metaphase for the induction of numerical and structural changes of chromosomes.

Chromosome identification is essential for biotechnolo-

gical studies including genome analysis, somatic hybridization and ploidy manipulation (Yamamoto and Tominaga, 2004). Since chromosomes are physical entity of the genetic system it is naturally assumed that any agent affecting the chromosomes will also lead to heritable genetic change.

As the sizes of chromosomes in *A. cepa* is comparatively larger than many other plants and eukaryotes and the number of chromosomes is less (2n =16), Grant (1982) reported *Allium* test as one of the excellent assay for mutagenesis assay. The common onion is one of the most outstanding higher plant recommended by United States Environmental Protection Agency (USEPA) and the American Society for Testing and Materials (ASTM) in 1982 and 1994 respectively for use as an excellent and alternative first-tier indicator for safety evaluation of cytogenetic and mutagenic effects of drinking water and environmental pollutants as their root length inhibition and chromosome aberration bioassay are sensitive, cost effective and valid indicator of toxicity test for routine monitoring of water pollution having good correlation with other test systems involving genotoxicity (Rank, 2003; Babatunde and Bakre, 2006; Olorunfemi et al., 2011). The test is a fast and inexpensive method, easy to handle, gives reliable results, comparable with other tests performed in mammalian systems is in high concurrence with similar assay in bone marrow cells in rats (Grover et al., 1998).

The Reactive Turquoise Blue is a chemical of regular use in small scale cottage industries of cotton fabrics in various parts of India with special reference to Western and Southern Odisha for handlooms works, but its genotoxicity due to waste water discharged on surrounding biomass in terms of any cytological end point has not been analyzed till to date. The purpose of our study is to evaluate the genotoxic effect of reactive turquoise blue (Procion MX) under various concentrations by analyzing mitotic aberrations in growing root tip cells of *A. cepa*.

MATERIALS AND METHODS

Test dye

The material used in this study is a dye that is, reactive turquoise blue (Procion MX) which is assumed to be a potential genotoxicant on plant model. The dye was purchased from the local market of Bargarh, Odisha, India which is used mostly by the weaver community residing in nearby areas for tie and dye techniques. It is a product of Atul Textiles and Imperial Chemical (ATIC) industry, Rajasthan, India.

Experimental plant organism

The experimental organism employed was *A. cepa.* It is one of the most extensively used biennial plants (Kovatch, 2003) and oldest cultivated vegetables (Fritsch and Friesen, 2002; Phillip and Jenderek, 2003). Cytotoxicity and environmental pollution (El-Shahaby et al., 2003) have been assessed by *A. cepa* root tip system, which is known to give similar results to *in vivo* animal cytotoxicity tests (Teixeira et al., 2003; Vicentini et al., 2001).

Methods

Various concentrations of the dye (weight per volume) in distilled water (0.01, 0.05, 0.1, 0.5 and 1.0%) were prepared and applied to the growing root tips of *A. cepa* (N = 6 to 10 for each treatment) in separate glass tubes of 10-20 ml capacity and incubated for various durations that is, 48 h to induce mitotic aberrations *in vivo* and for 24 and 48 h to assess mitotic index by recording the proportion of dividing cells. The procedure involved original form of *A. cepa* test (Fiskesjso, 1985) where root growth was initiated in tap water. Standard protocol (Fiskesjo, 1985) was followed with slight modification in the treatment schedule for duration of exposure and concentration of dye (w/v). The method used was similar to the method of Asita and Matebesi (2010).

The bulbs presoaked in distilled water were subsequently germinated in sand trays and grown *in situ* in different test tubes containing various concentrations of dye for 24 and 48 h. The test tubes with diluted test dye in distilled water for each bulbs were filled every day to compensate evaporation. When the root growth reached the length of 1 to 2 cm the tips were cut, fixed and preserved. The cut root tips were fixed in 1:3 aceto-alcohols (Carnoy's fixative) for 24 h and then stored in 70% alcohol for future use. The bulbs grown in tap water served the purpose of control. No positive control experiment employing mitomycin-c or cyclophosphamide was performed. Conventional squash preparation was adopted following the acid hydrolysis of cellulosic cell wall in 1 N HCl followed by warming at 60°C. Staining was done in 2% aceto-carmine in 45% glacial acetic acid (v/v) followed by rubbing (mordenting) in rust free iron needle to visualize the scorable stages under microscope (Das, 1986; Kar, 1992; Rank and Nielsen, 1997; Dane and Dalgic, 2005; Tartar et al*.,* 2006).

Scoring

The slides were viewed under the binocular light microscope (Olympus CX 31) using the 100 X objective lens with oil immersion. Photographs of some representatives selected stages were taken by a 14.2 mega pixel Canon Cyber Shot Digicam. A total of 300-700 cells were scored per slide mounting to an average of 3979 cells (2220 to 4717). Mitotic indices (MI) and mitotic depression (MD) were calculated following the procedure of Das (1986) and Kar (1992). The mitotic index (MI) in percentage was calculated as number of dividing cells / total number of cells scored × 100. Similarly, the mitotic depression (MD) was calculated as {MI (control)-MI (treated) / MI (control)} × 100. The proportion of specific cell abnormalities such as abnormal prophase, metaphase and anaphase was calculated in terms of percentage of the type of abnormality out of the total number of cells counted.

Statistical analysis

The mean value with standard deviation (SD) for each root length was calculated from values obtained from individual bulbs and it was compared with the corresponding control values and student's 't' test was conducted to ascertain if the differences were statistically significant or not for root growth and for total cell abnormality.

RESULTS AND DISSCUSSION

This study was based on two parameters namely-

Table 1. Effect of Reactive Turquoise Blue on MI and MD in root tip cells of *A. cepa*

Parameter	C	T1	T2	T3	T4	T5
Concentration (mg/L)	0	1.0	0.5	0.1	0.05	0.01
Duration (h)	48	48	48	48	48	48
Number of Bulbs	4	6	3	5	3	3
Number of cells scored (N)	4413	2220	4508	4000	4020	4717
Number of dividing cells (n)	160	45	91	159	163	218
Mitotic Indices (MI)	3.62	2.02	2.01	3.97	4.05	4.62
Mitotic depression (MD)	_	44.19	44.47	-9.66	-11.87	-27.62

C= Control in distilled water; Mitotic index (MI) = n/N X100; Mitotic Depression (MD) = [MI (Control)-MI (Treatment)/MI (Control)] X100. T_1, Treatment 1 of onion root tips at a concentration of 1.0 mg/L of Reactive Turquoise Blue dye; T_2, treatment 2 of onion root tips at a concentration of 0.5 mg/L of Reactive Turquoise Blue dye; T3, treatment 3 of onion root tips at a concentration of 0.1 mg/L of Reactive Turquoise Blue dye; T_4, treatment 4 of onion root tips at a concentration of 0.05 mg/L of Reactive Turquoise Blue dye; T_5, treatment 5 of onion root tips at a concentration of 0.01 mg/L of Reactive Turquoise Blue dye.

Table 2. Root growth (mm) in *A. cepa* bulbs treated with Reactive Turquoise Blue (Procion MX) (Mean± SD).

Treatment	C1	C2	T1 (A)	T1(B)	T2 (A)	T2 (B)	T3 (A)	T3 (B)	T4(A)	T4 (B)	T5(A)	T5(B)
Conc. (mg/L)	0	0	1.0	1.0	0.5	0.5	0.1	0.1	0.05	0.05	0.01	0.01
Duration (h)	24	48	24	48	24	48	24	48	24	48	24	48
Number of Bulbs	4	4	7	6	5	3	5	5	4	3	3	3
Mean Root Length ± SD	5.80 ± 0.35	6.00 ±0.44	5.40 ± 2.16	5.80 ± 1.28	5.90 ± 0.84	7.40 ± 1.80	6.60 ± 1.42	6.80 ± 2.27	4.90 ± 1.06	6.30 ± 0.24	4.10 ± 0.56	5.40 ± 1.36
t values												
(p=0.005)	NA	NA	NS	NS	NS	*	NS	*	NS	NS	NS	NS
(p=0.05)			NS	NS	NS	**	NS		NS	NS	NS	NS

C_1, Control-1; C_2, Control-2; * Significant at p=0.005; ** Significant at p=0.05; NS, not significant; NA, Not Applicable. T_1, Treatment 1 of onion root tips at a concentration of 1.0 mg/L of Reactive Turquoise Blue dye; T_2,Treatment 2 of onion root tips at a concentration of 0.5 mg/L of Reactive Turquoise Blue dye; T3, Treatment 3 of onion root tips at a concentration of 0.1 mg/L of Reactive Turquoise Blue dye; T_4, Treatment 4 of onion root tips at a concentration of 0.05mg/L of Reactive Turquoise Blue dye; T_5, Treatment 5 of onion root tips at a concentration of 0.01 mg/L of Reactive Turquoise Blue dye.

cytogenetic and root growth. It was observed that, at higher concentrations that is, - 1.0 and 0.5%, the onion root tip cells could not survive and died in 24 h causing the tips to dry. The dried root tips were presumed to be due to death of cells caused by evaporation of the liquid or cytotoxic nature of chemical. The tips became hard, rough and difficult to cut for preservation. The results are presented in form of observation (Tables 1 to 3, Figures 1 to 5 and Plates 1 to 6).

Table 1 as well as Figures 1 and 2 describe the effect of dye on MI and MD. An average of 3-6 bulbs were used for each treatment and the number of cells scored (N) for MI calculation ranged from 2220 to 4717 (mean = 3979). The

Table 3. Percentage of abnormal mitotic cells induced by Reactive Turquoise Blue.

Treatment number	C	T1	T2	T3	T4	T5
Concentration (mg/L)	0.0	1.0	0.5	0.1	0.05	0.01
Duration (h)	48	48	48	48	48	48
Abnormal Cells (%)	0.11	1.48	3.20	2.42	2.81	3.18
% of Abnormality						
A.P	0.00	0.31	0.59	0.62	0.59	0.73
A.M	0.04	0.49	0.68	0.92	1.16	1.34
A.A	0.02	0.63	0.37	0.70	0.79	0.96
A.T	0.04	0.04	0.24	0.10	0.24	0.12
t values for total cell abnormality						
(p=0.005)	NS	NS	*	NS	NS	*
(p=0.05)	NS	NS	NS	NS	NS	**
Bi or multi nucleated cells	0.00	0.00	1.48	0.02	0.00	0.00

C, Control; AP, abnormal prophase; AM, abnormal metaphase; AA, abnormal anaphase; AT, abnormal telophase. T_1, Treatment 1 of onion root tips at a concentration of 1.0mg/L of Reactive Turquoise Blue dye; T_2, Treatment 2 of onion root tips at a concentration of 0.5 mg/L of Reactive Turquoise Blue dye; T3, Treatment 3 of onion root tips at a concentration of 0.1 mg/L of Reactive Turquoise Blue dye; T_4,Treatment 4 of onion root tips at a concentration of 0.05 mg/L of Reactive Turquoise Blue dye; T_5, Treatment 5 of onion root tips at a concentration of 0.01mg/L of Reactive Turquoise Blue dye.

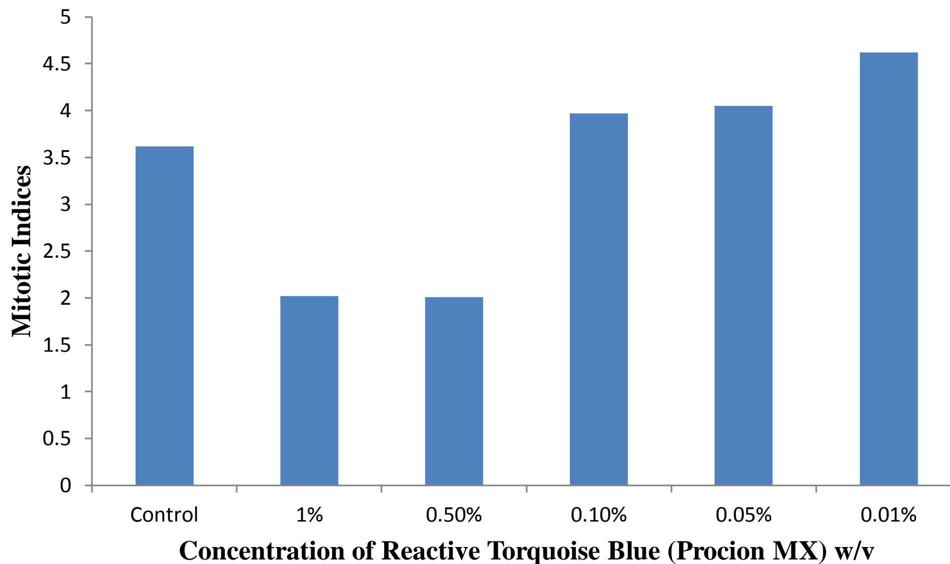

Figure 1. Effect of reactive turquoise blue on mitotic indices (MI) of *A. cepa* root tip cells.

maximum number of cells scored was in the case of treatment with lowest concentration that is, 0.01 mg/L. Highest number of dividing cells (n) was observed (218) with largest MI value of 4.62 in the above treatment. Similarly, the lowest number of dividing cells were observed at 1.0 mg/L (N=2220, MI=2.02) but minimum MI (2.01) was calculated at 0.5 mg/L. A gradual increase with decreasing concentration was observed.

The mean root length varied from 4.10 ± 0.56 to 7.40 ± 1.80 mm (N=6 to 10) and presented in Table 2 and Figure 3. After 24 h, maximum root length was observed at 0.1 mg/L (6.60 ± 1.42 mm) in three replications of 25 each and minimum root length was 4.10 ± 0.56 mm at 0.01 mg/L in three replications of 25 each. Similarly at 48 h treatment schedule, maximum root length was observed (7.40 ± 1.80 mm) at 0.5 mg/L and minimum root length was 5.40 ± 1.36 mm at 0.01mg/L.

Table 3 and Figure 4 show the degree of abnormality of cells at various treatment schedules. Usually under microscope, abnormal divisional stages appeared in

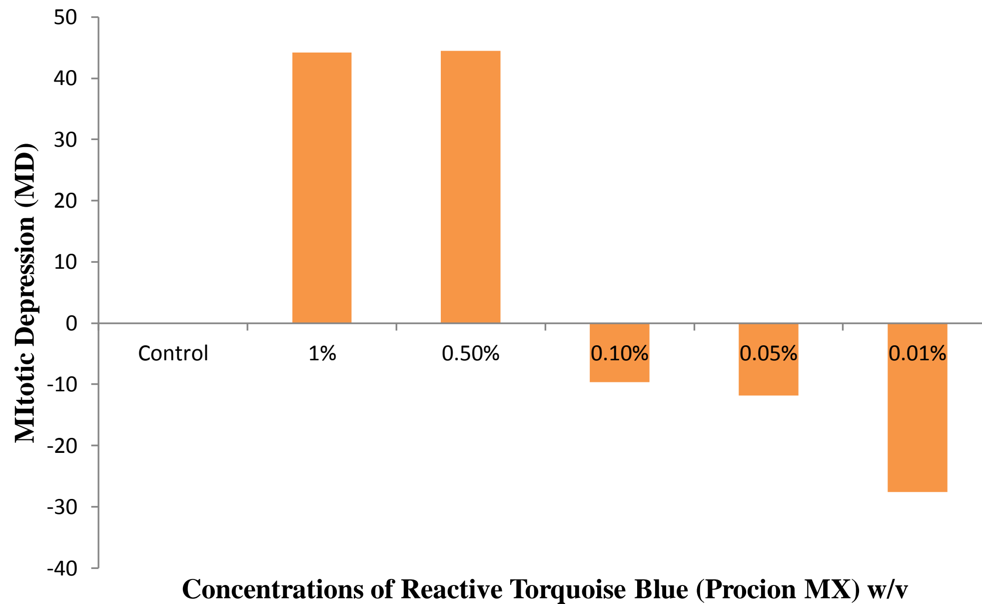

Figure 2. Mitotic depressions (MD) in *A. cepa* root tip cells induced by Reactive Turquoise Blue.

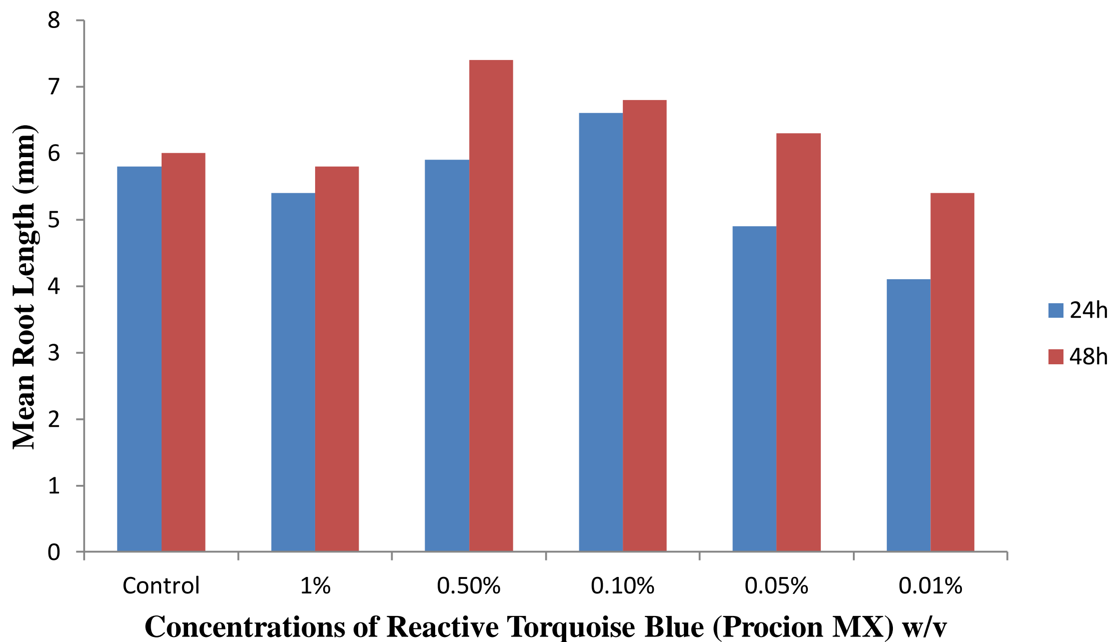

Figure 3. Mean root length (mm) in *A. cepa* due to effect of Reactive Turquoise Blue for 24 and 48 h.

wrong geometric locations and orientations with respect to the equatorial plate of the rectangular or square type cells. Normal prophase or metaphase appeared symmetrical under 100 X magnification with respect to cell boundary and the staining of the nuclear region differed from that of the cytoplasm. Maximum of 3.2% of total abnormal cells were observed at 0.50% and minimum total abnormality observed were 1.48% at a concentration of 1%. The t values for total cell abnormality presented in Table 3 indicated significant deviation from control at 0.5 mg/L at probability level of 0.005 only and significant at 0.01 mg/L at both 0.005 and 0.05 level of probabilities.

Figure 5 presents each type of abnormality in *A. cepa* root tip cells due to different treatment of dye for 48 h. A

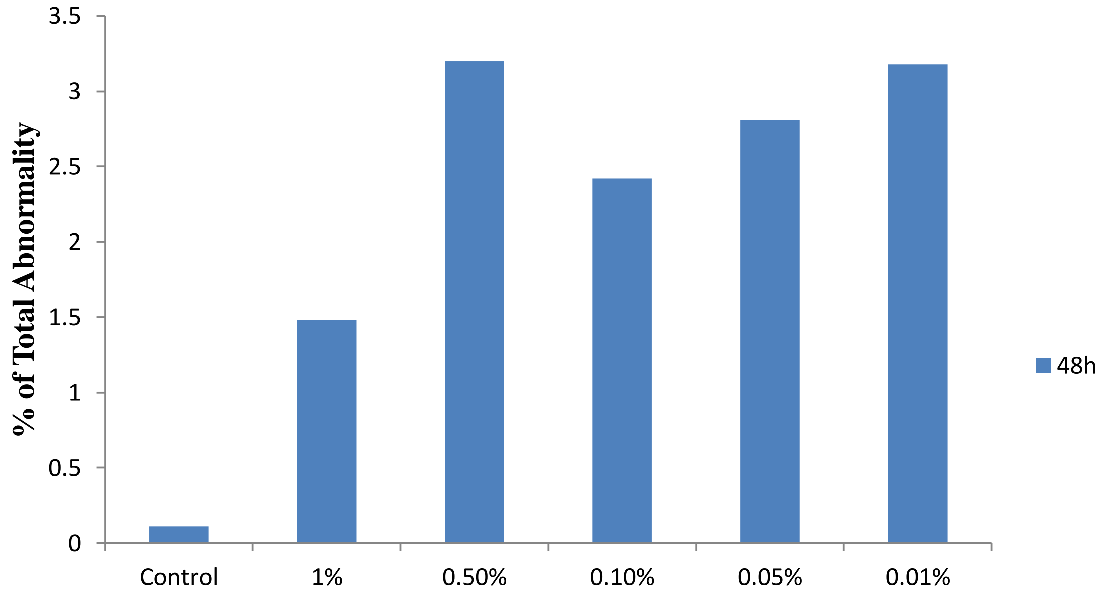

Figure 4. Total abnormality in *A. cepa* root tip cells (%) induced by Reactive Turquoise Blue.

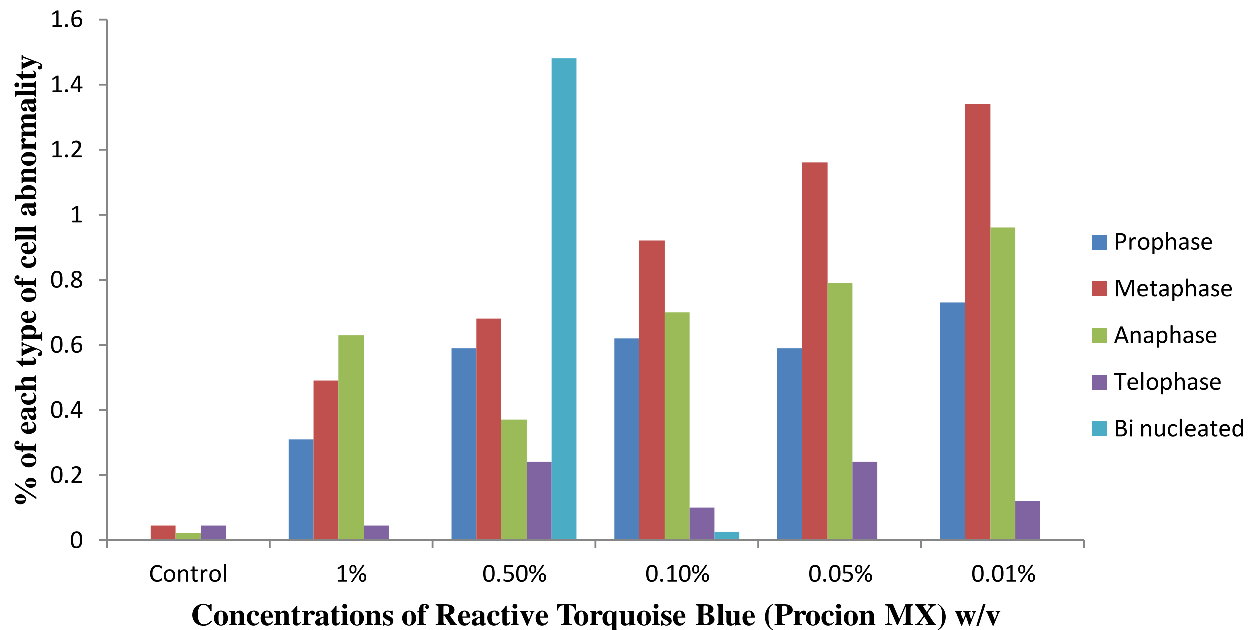

Figure 5. Each type of mitotic aberration (%) in *A. cepa* root tip cells due to Reactive Turquoise Blue.

good correlation was observed between the concentrations of dye and percentage of abnormal metaphase showing an inverse relationship.

Percentage of abnormal prophase and metaphase gradually increased with decreasing concentration of the dye; but, irregular pattern of abnormal anaphase and telo-phase was observed with decreasing concentration. In this study, the t test for root growth observed was found to be insignificant in many cases in comparison to those at control experiments at 0.05 as well as 0.005 level of probability.

Only in case of treatment at 48 h at 0.5% concentration

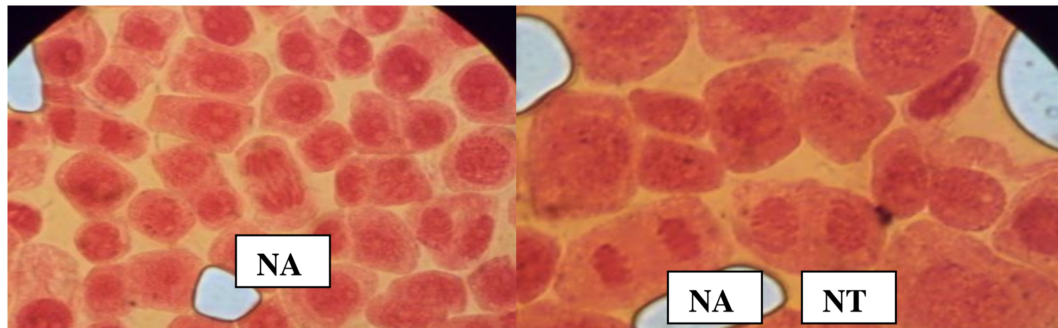

Plate 1. Stages of mitosis in root tip cells of *A. cepa* in control. NA: normal anaphase, NT: normal telophase.

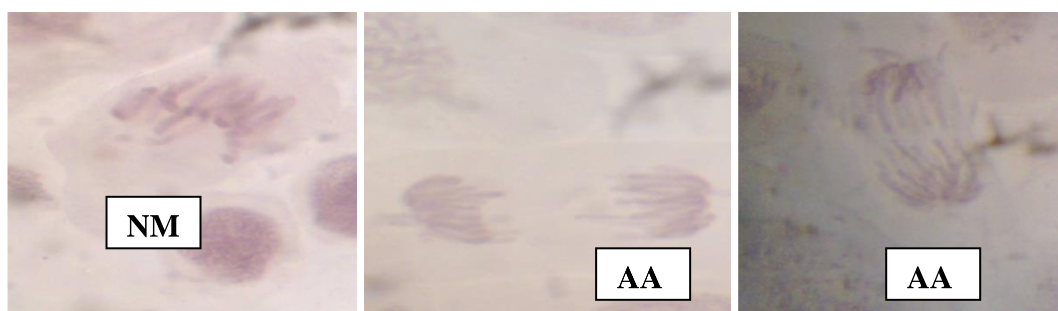

Plate 2. Mitotic aberrations in root tip cells of *A. cepa* in Reactive Turquoise Blue at 0.01%. NM, normal metaphase; AA, abnormal anaphase.

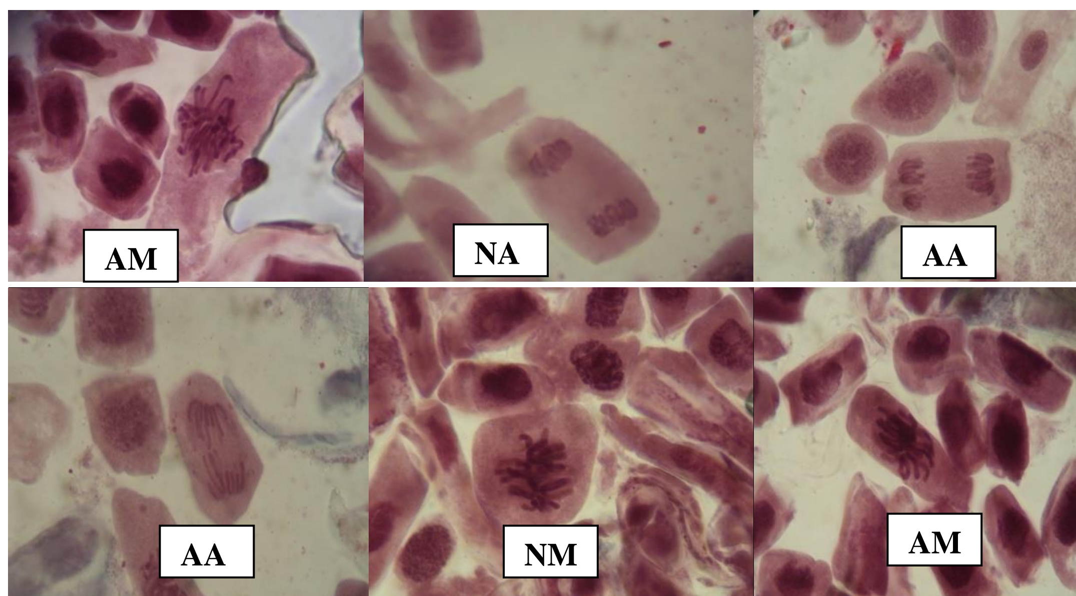

Plate 3. Mitotic aberration in root tip cells of *A. cepa* in Reactive Turquoise Blue at 0. 05%. AM, abnormal metaphase; NA, normal anaphase; AA, abnormal anaphase; NM, normal metaphase.

of the dye the t value showed significant deviation from control value at both 0.05 and 0.005 level of probability.

However, at a concentration of 0.5% of the dye, 1.48% of bi- nucleated cells were observed in comparison to 0.025% of bi-nucleated cells at a concentration of 0.1%.

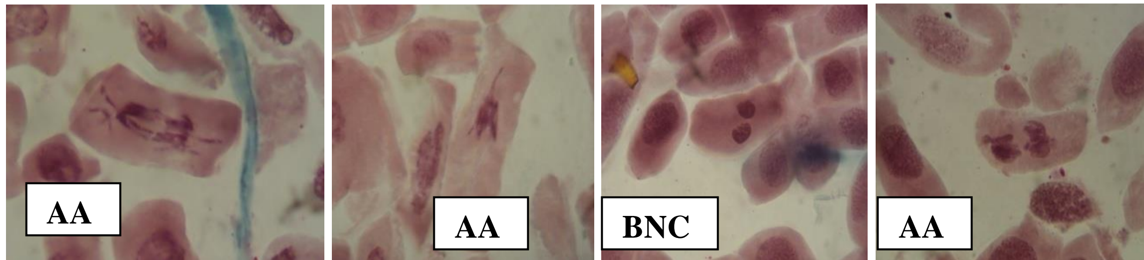

Plate 4. Mitotic aberrations in root tip cells of *A. cepa* in Reactive Turquoise Blue at 0.1%. AA, abnormal anaphase; BNC, Bi-nucleated cells.

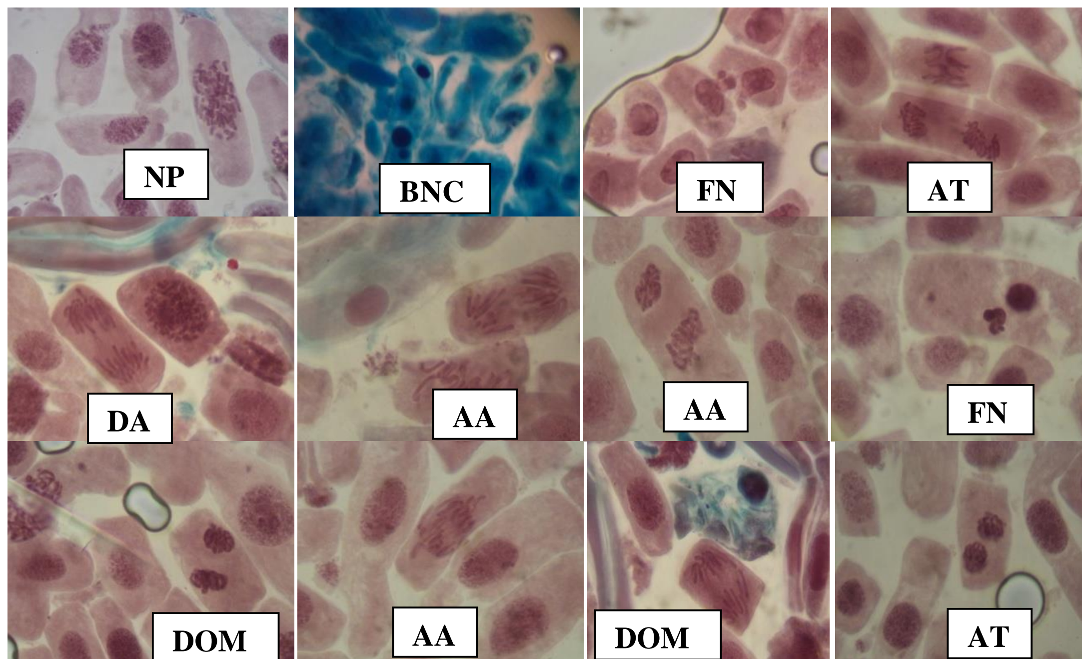

Plate 5. Mitotic aberrations in root tip cells of *A. cepa* in Reactive Turquoise Blue at 0.5%. NP, normal prophase; BNC, Bi-nucleated cells; FN, fragmented nucleus; AT, abnormal telophase; DOA, disoriented anaphase; DOM, disoriented metaphase; AA, abnormal anaphase.

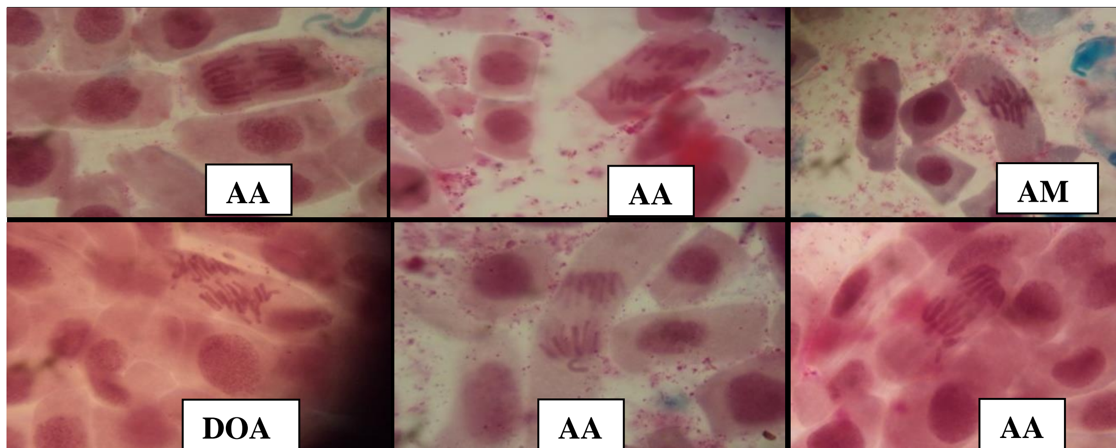

Plate 6. Mitotic aberrations in root tip cells of *A. cepa* in Reactive Turquoise Blue at 1.0%. AA, abnormal anaphase; AM, abnormal metaphase; DOA, disoriented anaphase.

DISCUSSION

Reactive dyes are highly water soluble and are non-degradable in the conventional, biological treatment systems, adsorb poorly to biological solids and therefore remain in the discharged effluents (Epolito et al., 2005). The synthetic dyes used in textile industries are of health concern for various organisms including human. Rajaguru et al. (2001) reported a moderately toxic dye to *Rana hexadactyla* following whole-body exposure to increasing concentrations. The female crab *Spiralothelphusa hydrodroma* after exposure to textile dye effluents at a sub-lethal concentration (66.69%) in two different exposure period shows morphological as well as histological changes in neuro-secretory cells of brain, thoracic ganglia and eyestalks and their contents (Sekar et al. 2008). Birhanli and Ozmen (2005) used frog embryo teratogenesis assay-Xenopus (FETAX) to establish that some reactive dyes have teratogenic potential. Risk of colon and rectum cancers relates mostly to dyes for synthetic fibres (De Roos et al., 2005).

Sahi et al. (1998) used *A. cepa* test system to assess the effects of chromium contamination in the water of an Indian river and showed that at sites where chromium concentration was high there was a reduction in mitotic index and an increase in the rate of mitotic abnormalities, thus confirming the cytotoxic and genotoxic effect of chromium. Similar trends were also observed in the present study (Table 1 and Figure 1). Mitotic activity reduction could be due to the inhibition of DNA synthesis (Sudhakar et al., 2001). In the present study, it was observed that at 0.5 and 1.0% of concentrations, the root tips were dry due to death of cells. The deaths of root cells at such higher concentrations were assumed to be due to interferences of dye in regular cellular activities making them cytotoxic. However in lower concentrations, such deaths of root tip cells were not prominent.

The present study is comparable to similar other studies done earlier by various groups of workers in different non-human organisms (Badr, 1983; Das, 1986; Jain and Sarbhoy, 1988; Kar, 1992; El-Shahaby et al., 2003; Wollin and Gorlitz, 2004). Presence of chromosomal bridges at anaphase might be the result from chromosome stickiness as reported by Badr (1983) caused by clastogenecity. In the present study, the dye affected cell plate formation, which was found to be under great disturbances and the orientation as well as functioning of the spindle was disturbed. In several late anaphases, one group of chromosomes was found to take the extreme terminal position while the other was in the middle. Similar orientations of the two nuclei were also noticed in certain bi-nucleated cells. Occurrence of disoriented chromosomes might have been brought about by action of the dye on the microtubules. The dye might have caused the failure of chromosomes to align at equatorial plate because of the dysfunction of spindle and energy deficiency causing delay in the division of centromeric region which might have also caused distorted chromosome as reported earlier (Jain and Sarbhoy, 1988). Irregular and transverse orientations of chromosomes in equatorial plate were also observed as common form of mitotic aberration. In metaphase, chromosomes were arranged in equatorial plate which was diagonally placed. In anaphase, chromosomal bridge and orientation problem in pole was observed.

The condensation of interphase chromatin to form the compact chromosomes of the mitotic cells is a key event in mitosis, critical in enabling the chromosomes to move along the mitotic spindle without becoming being broken or entangled with one another. The chromatin in interphase nuclei condenses nearly thousand fold during the formation of metaphase chromosome. Cooper and Haussmann (2007) informs that, chromatin condensation is driven by protein complexes called condensins which are members of a class of 'structural maintenance of chromatin proteins' (SMC) that play key role in organization of eukaryotic chromosomes. Another family of SMC proteins called cohesins contributes to chromosome segregation during mitosis. The SMC proteins are recognized as one of the most fundamental classes of proteins that regulate the structural and functional organization of chromosomes from bacteria to humans (Losada and Hirano, 2005; Nasmyth and Haering, 2005).

The final conclusion of this study includes observations of abnormally de-condensed or elongated or stretched chromosomes in numbers of prophases, metaphases and anaphases. Those are considered to be due to disturbance of chromosomal condensation mechanism by the genotoxicant. Disoriented mitosis, decrease in mitotic indices, abnormal divisional stages, and orientation problems of equatorial plate indicating chromosomal disturbances with respect to geometrical orientation indicates genotoxicity of the chemical Reactive Turquoise Blue (Procion-MX). However, further in-depth study involving various chromosomal banding techniques or involvement of molecular markers with protein or nucleic acid specific hybridization after treating with the chemicals in *A. cepa* root tip cells as well as mammalian and non-mammalian cell systems can give further evidence with firm support to prove the cytotoxicity as well as genotoxicity of dye. This may be considered as a preliminary information to create public awareness regarding commercialization, large scale utilization and management as well as disposal of similar potentially genotoxic chemical to the environment.

Conflict of Interests

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

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Full Length Research Paper

In vitro propagation of garlic (*Allium sativum* L.) through adventitious shoot organogenesis

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The present study investigates *in vitro* regeneration of three garlic cultivars; that is, Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type through adventitious shoot organogenesis. Shoot and root apices were subjected to eight callus induction treatments. A combination of 1 mg L^{-1} 2,4-D + 5 mg L^{-1} BA + 5 mg L^{-1} NAA produced a 100% of callus induction from root apices for all garlic cultivars tested. Shoot apices showed higher frequency of callus induction than root apices. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. Shoot apices had higher callus fresh weight than root apices in Balady and Sids 40 cultivars as well as wild type. The cultivar Balady had the highest callus fresh weight whereas the wild type had the lowest values. There were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. The wild type failed to regenerate shoots. The highest shoot number per root induced callus (48.8) was obtained from C7 callus line cultured into B5 medium supplemented with 10 mg L^{-1} Kin and 2 mg L^{-1} IAA. The cultivar Sids 40 showed the lowest regeneration efficiency among other cultivars with 1.5 shoots per explant. For callus induced from shoot apices, the eight callus lines for both Sids 40 cultivar and the wild type failed to regenerate shoots and the cultivar VFG 180 (3 -1) showed very low regeneration efficiency. The Balady cultivar showed the highest regeneration efficiency with 39 shoots per explant. The regenerated garlic shoots were *in vitro* rooted and acclimatized in greenhouse prior to their cultivation in open field. Garlic plantlets derived through tissue culture required three vegetative generations to produce bulbs of commercial size.

Key words: Acclimatization, garlic, organogenesis, tissue culture.

INTRODUCTION

Garlic (*Allium sativum* L., Liliaceae) is an important and widely cultivated crop, which is known for its culinary and medicinal use. Garlic has been cultivated vegetatively because of its sexual sterility (Etoh, 1985). Vegetative

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **Kin,** kinetin; **IAA,** indole-3-acetic acid; **BA,** 6-benzyladenine; NAA**,** naphthalene acetic acid.

Table 1. Main characters for the investigated garlic cultivars (Balady, Sids 40 and VFG 180 (3-1)) and an Egyptian wild type.

Characters Source	*Allium sativum* L.			Egyptian wild type
	Balady	**Sids 40**	**VFG 180 (3-1)**	
	Egypt	**Egypt**	**Taiwan**	**North region of Egypt**
Plant height (cm)	104.4±2.09a[Z]	65.5±1.64d	85.2±1.70c	96.5±2.51b
Pseudostem length (cm)	41.3±1.65a	28.0±0.56c	34.4±0.71b	17.5±0.53d
No. of leaves/plant	13.0±0.57a	10.3±0.31b	8.8±0.30b	9.5±0.35b
5th leaf length (cm)	62.0±1.24a	39.3±0.98b	58.0±1.16a	33.5±0.87c
5th leaf width (cm)	1.6±0.05c	3.7±0.13a	4.1±0.16a	2.5±0.10b
Total yield (ton/fed.)	5.3±0.23b	5.6±0.17b	8.3±0.28a	1.2±0.04c
Bulb weight (g)	76.4±2.29b	73.1±1.97b	117.4±2.35a	18.0±0.37c
Bulb diameter (cm)	5.8±0.12b	5.1±0.15b	7.7±0.21a	2.5±0.06c
No. of cloves / bulb	55.2±1.13a	17.0±0.51b	16.5±0.33b	2.0±0.03c
Clove weight (g)	1.38±0.05d	4.3±0.12c	7.2±0.15b	8.5±0.22a
Bulb skin color	White	Purple	Purple	Cream
Bulblets (helmet-shaped)	-	-	-	Many small with stolon from stem disk
Bulbils	Few small	Few small to medium	Few medium	-
Number of chromosomes*	16	16	16	32

*The cytological study was carried out according to Darlington and La Cour (1976) technique. [Z]Data presented are means ± standard error. Mean separation within rows by Duncan's multiple range tests at 5% level.

propagation of garlic is achieved through division of the ground bulbs and/or aerial bulbs therefore, the multiplication rate is fairly low. Also, due to difficulties of inducing flowering, improvement of this crop through breeding programs is limited (Barandiaran et al., 1999a; Metwally et al., 2014). Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect pests (Verbeek et al., 1995). Virus infection was shown to reduce the bulb yield by 20 to 60%, and up to 80% in case of mixed infection, depending on cultivar and stage of infection (Lot et al., 1998). The virus infection is inevitable for vegetative propagation of garlic. Therefore, *in vitro* propagation would be one of the key technologies for sustainable supply of this important plant source

The low propagation rate and the continuous accumulation of deleterious viruses produced in the field have promoted the development of *in vitro* propagation of garlic (Nagakubo et al., 1993; Seabrook, 1994; Koch et al., 1995; Mohamed-Yassen et al., 1995; Haque et al., 1997, 2003; Ayabe and Sumi, 1998; Myers and Simon, 1998, 1999; Robledo-Paz et al., 2000; Kim et al., 2003; Luciani et al., 2006; Keller and Senula, 2013). According to these reports, the physiological condition of the explant, the genotype and the growth regulator combinations used in the culture medium were the most important factors affecting plant regeneration. However, several months up to one year are required for plant regeneration; therefore, the efficiency of plant regeneration has not been reproducibly high. In continuation to improve *in vitro* culture and propagation for garlic cultivars, in the present study, a detailed investigation was carried out in order to establish *in vitro* culture protocol for three garlic cultivars; that is Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type.

MATERIALS AND METHODS

Plant material and culture establishment

The present study was conducted on three cultivars of garlic (*A. sativum* L.); that is Balady, Sids 40 and VFG 180 (3 - 1) as well as an Egyptian wild type collected in 2009 from El-Arish city at the North region of Sinia peninsula. The main vegetative characteristics as well as chromosomal number for these garlic genotypes are presented in Table 1 and Figure 1. Garlic cloves were separated from the compound bulb and pealed manually, washed and air dried. Small and injured cloves were excluded. The cloves were rinsed in 70% (v/v) ethanol for 30 s followed by 3.5% sodium hypochlorite containing one drop of Tween 80 for 20 min under a constant hand agitation. Cloves were then washed thrice with sterile distilled water for 5 min each. The cloves were inoculated in sterile glass jars of 100 ml each containing 20 ml of half strength MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). Root and shoot tips of the sprouting plantlets were used as plant material for callus induction. Root tips were 0.8 to 1.2 cm in length whereas shoot tips were 1.0 to 1.5 mm in length.

Culture conditions

All media were supplemented with 3% (w/v) sucrose and 0.8% (w/v) Sigma agar-agar. The pH of the medium was adjusted to 5.8 before

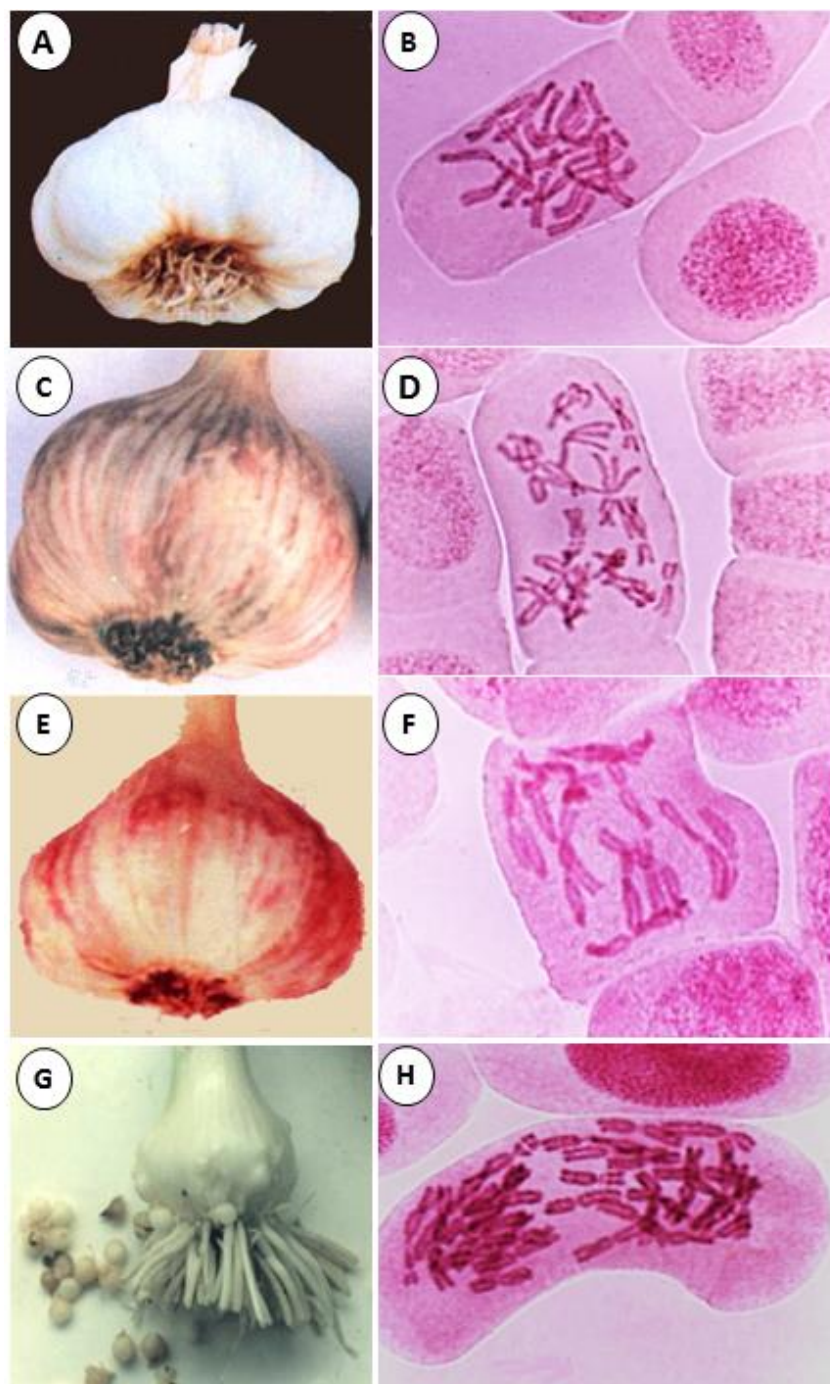

Figure 1. Morphological characteristics and chromosomal number in garlic cultivars. **A, B)** Balady; **C, D)** Sids 40; **E, F)** VFG 180 (3 - 1) and **G, H)** wild type.

autoclaving for 20 min at 121°C. The cultures were incubated for three days under dark conditions at 25 ± 2°C then under 16 h light at 35 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by cool white fluorescent tubes.

Callus induction

Root and shoot apices were cultured on either MS medium supplemented with different PGRs concentration and combinations [1.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) + 2.0 mg L^{-1} Kinetin (Kin) + 1.7 mg L^{-1} indole-3-acetic acid (IAA); 2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} 6-benzyladenine (BA); 5 mg L^{-1} BA + 5.0 mg L^{-1} 1-naphthalene acetic acid (NAA); 1 mg L^{-1} NAA; 3 mg L^{-1} 2,4-D; 1 mg L^{-1} 2,4-D; 1 mg L^{-1} 2,4-D + 5 mg L^{-1} BA + 5 mg L^{-1} NAA] or B5 medium (Gamborg et al., 1968) supplemented with 3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} 2ip. Culture conditions are as described above. There were four explants per replicate and four replicates per treatment

Table 2. Frequency of shoot and root apices induced callus of three garlic cultivars {Balady, Sids 40 and VFG 180 (3 - 1)} as well as wild type after 8 weeks of culture.

Medium code[Y]	Balady	Sids 40	VFG 180 (3 - 1)	Wild type
Root apex				
C1	100a[Z]	90.6b	100a	100a
C2	100a	100a	85c	68d
C3	100a	20g	100a	100a
C4	0h	0h	100a	0h
C5	100a	100a	25.8g	76d
C6	100a	100a	42.9f	100a
C7	100a	100a	100a	100a
C8	100a	30g	100a	100a
Shoot apex				
C1	100a	91.7b	100a	100a
C2	100a	66.7d	68.8d	100a
C3	100a	50.0f	100a	100a
C4	94.5b	91.7b	100a	90b
C5	100a	66.7d	62.5e	70d
C6	100a	58.3e	93.8b	100a
C7	100a	41.7f	87.5c	70d
C8	100a	50.0f	100a	100a

[Y]Callus induction media were supplemented with various PGRs as follows: C1 (1.1 mg l^{-1} 2,4-D + 2.0 mg l^{-1} Kin + 1.7 mg l^{-1} IAA); C2 (2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA); C3 (5 mg l^{-1} BA + 5.0 mg l^{-1} NAA); C4 (1 mg l^{-1} NAA); C5 (3 mg l^{-1} 2,4-D); C6 (1 mg l^{-1} 2,4-D); C7 (1 mg l^{-1} 2,4-D + 5 mg l^{-1} BA + 5 mg l^{-1} NAA); C8 (3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2ip). [Z]Mean separation within columns by Duncan's multiple range test at 5% level.

rendering a group of 16 explants per treatment. The percentage of callus formation and callus fresh weight were recorded after eight weeks of culture.

Adventitious shoot regeneration

All calluses were transferred onto either MS medium supplemented with different concentrations and combinations of BA and NAA as follows: BA (1.0 mg L^{-1}) + NAA (0.5 mg L^{-1}); BA (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}); BA (2.0 mg L^{-1}) + NAA (1.0 mg L^{-1}) or B5 medium supplemented with Kin (10.0 mg L^{-1}) + IAA (2.0 mg L^{-1}). There were five explants per Petri dish and four replicates per treatment. Culture conditions are as described above. The number of proliferated shoots per callus was recorded after eight weeks of culture for Balady cultivar while after 12 months for Sids 40 and VFG 180 (3 - 1) cultivars.

Root formation and plant acclimatization

Proliferated shoots obtained through organogenic calli were individually separated and inoculated to cylindrical culture jars (375 ml capacity) containing 35 ml MS medium without PGRs. The medium was supplemented with 3% (*w/v*) sucrose and solidified with 0.8% (*w/v*) agar. The pH of the medium was adjusted to 5.8 before autoclaving (at 121°C and 1.2 kg cm^{-2} pressure for 15 min). All the culture jars were maintained for four weeks at the same culture conditions described above.

For pre-acclimatization of garlic plantlets, the leaves were trimmed and the plantlets were sub-cultured for four weeks. The jars were subjected to a light intensity of 35 μ mol m^{-2} s^{-1} PPF for 2 weeks followed by 50 μmol m^{-2} s^{-1} PPF for two weeks. The plantlets were carefully cleaned from the medium and washed with tap water. They were then transplanted into sterilized clay pots (20 cm diameter) containing a mixture of peat moss: silt: sand (1:1:1, v/v/v). The leaves were trimmed into half size and the pots were incubated in a growth chamber. The environment in the growth chamber was adjusted to 25 ± 1°C air temperature, 50 μmol m^{-2} s^{-1} PPF with a 16 h photoperiod provided by cool white fluorescent tubes and 40 to 50% relative humidity. The pots were covered with a clear polyethylene bags for the first week, gradually removed and grown for three weeks. The acclimatized plants were then transferred to a greenhouse for 6 weeks before their planting in an open field.

Experimental design and data analysis

Experiments were set up in a completely randomized design. Data were subjected to Duncan's multiple range tests using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

RESULTS AND DISCUSSION

Callus induction

Both shoot and root apices excised from *in vitro* plantlets of three garlic cultivars as well as wild type were subjected to eight callus induction treatments in the first series of experiments (Table 2). Shoot apices formed callus in all eight treatments and in all garlic cultivars

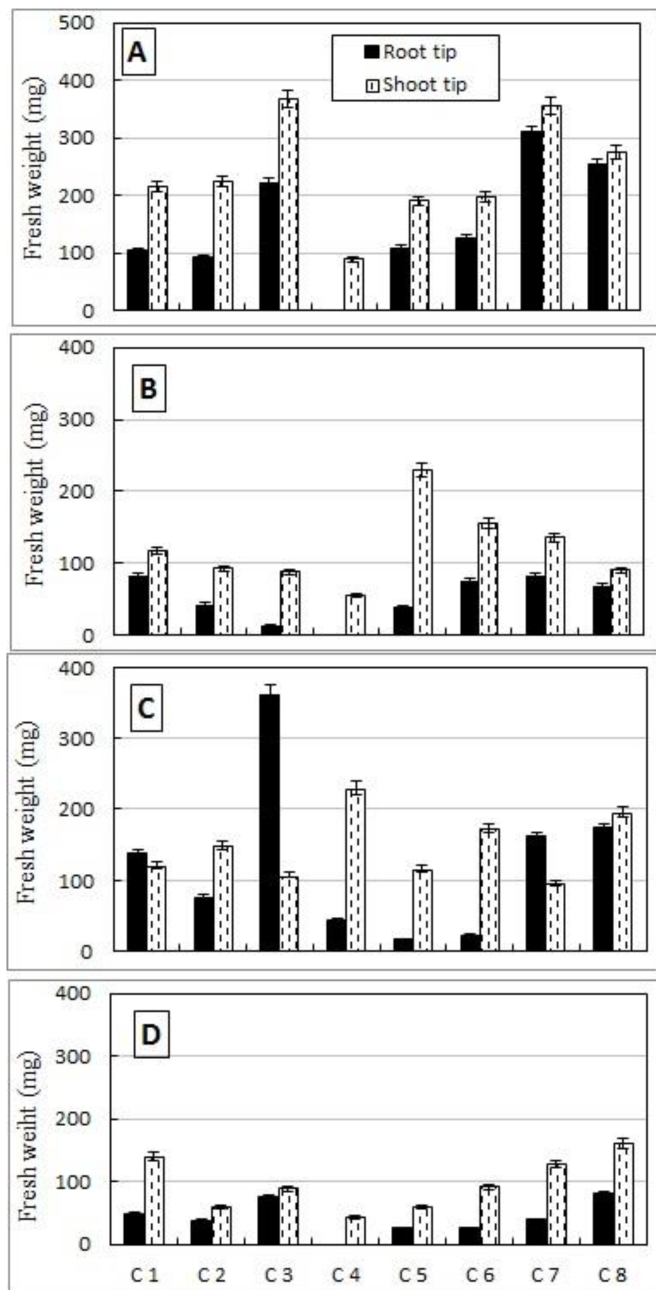

Figure 2. Fresh weight of root/shoot tips induced callus of Balady (A), Sids 40 (B), VFG 180 (3 - 1) (C) and wild type (D) after eight weeks on callus induction media supplemented with various PGRs: C1 (1.1 mg l^{-1} 2,4-D + 2.0 mg l^{-1} Kin + 1.7 mg l^{-1} IAA); C2 (2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA); C3 (5 mg l^{-1} BA + 5.0 mg l^{-1} NAA); C4 (1 mg l^{-1} NAA); C5 (3 mg l^{-1} 2,4-D); C6 (1 mg l^{-1} 2,4-D); C7 (1 mg l^{-1} 2,4-D + 5 mg l^{-1} BA + 5 mg l^{-1} NAA); C8 (3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2ip).

tested. Root apices also formed callus in all treatments except for C4 treatment which did not induce callus in Balady and Sids 40 cultivars as well as wild type. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. Shoot apices showed higher frequency of callus induction than root apices. There were differences among auxin/cytokinin callus induction treatments for the percentage of root and shoot apices that induced callus. A combination of 2,4-D, BA and NAA (C7 treatment) produced a 100% of callus induction from root apices for all garlic tested. For shoot apices, a combination of 2,4-D, Kin and IAA (C1 treatment) produced the highest frequency of callus induction at 100% for Balady, VFG 180 (3 -1) and wild type and 91.7% for Sids 40. In general, a high frequency of callus induction was obtained when auxin was combined with cytokinins.

The root and shoot apices of VFG 180 (3 -1) cultivar, contrary to the other garlic cultivars, produced a 100% of callus induction when NAA was employed alone without cytokinins. Garlic cultivars and different explants also showed variations for their callus fresh weight (Figure 2). Shoot apices had higher callus fresh weight than root apices in Balady and Sids 40 cultivars as well as wild type (Figure 2A, B and D). In VFG 180 (3 -1) cultivar, shoot apices also had higher callus fresh weight than root apices except for 3 callus induction treatments (C1, C3 and C7) (Figure 2C). In general, the cultivar Balady had the highest callus fresh weight whereas the wild type had the lowest values. Among the eight PGRs treatments, C3 (BA and NAA) produced the highest callus fresh from shoot apex in Balady cultivar and from root apex in VFG 180 (3 -1) cultivar. A combination of 2,4-D with BA and NAA (C7) or 2,4-D with 2ip (C8) also proved effective for callus fresh weight in Balady and VFG 180 (3 -1) cultivars as well as the wild type. Using auxins as the sole PGR in the medium such as 3 mg l^{-1} 2,4-D (C5) or 1 mg l^{-1} NAA (C4) produced the highest callus fresh weight from shoot apices in Sids 40 and VFG 180 (3 - 1), respectively. Previous reports demonstrated that callus differentiation in different garlic cultivars are determined by PGRs in the culture medium; 2,4-D was most effective (Myers and Simon, 1999; Robledo-Paz et al., 2000; Khar et al., 2005; Luciani et al., 2006).

In the present study, calluses were formed only on end of the root explant (Figure 3A). All calluses were compact and exhibited a nodular-like structure (Figure 3B). Previous reports demonstrated callus formation on the apical part of the root (Haque et al., 1998; Barandiaran et al., 1999b; Robledo-Paz et al., 2000). In contrary, it has been observed that calluses could be induced not only on the apical part of the root but also on the non-apical parts (Zheng et al., 2003).

Adventitious shoot regeneration from root apices induced callus

After callus induction, eight callus lines induced from root apices were transferred into four regeneration media rendering a group of 32 treatments for each cultivar as well as the wild type (Figure 4). In Balady cultivar, 25 out of 32 treatments produced shoots (Figure 4A). The

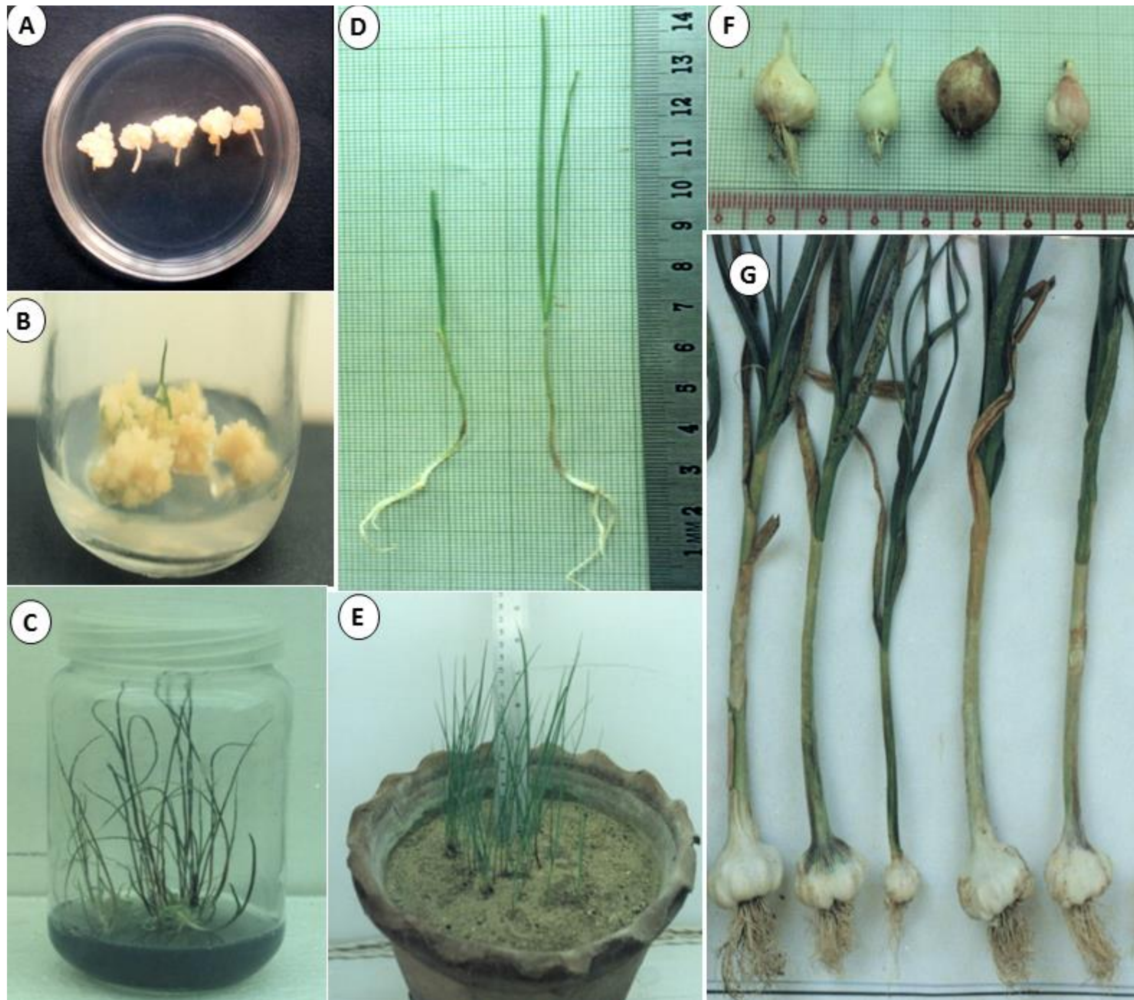

Figure 3. Indirect shoot organogenesis using root tip and development of garlic bulbs during three successive generations in Balady cultivar. **A)** Calluses formed on end of the root explants. **B)** Nodular meristematic callus. **C)** Regenerated garlic shoots. **D)** *In vitro* rooted plantlets. **E)** Acclimatized plantlets. **F)** Non-divided bulbs derived from the first vegetative generation in pots. **G)** Bulbs derived from the third vegetative generation in the field.

highest shoot number per explant (48.8) was obtained from C7 callus line cultured into R4 regeneration medium (B5 + 10 mg L^{-1} Kin + 2 mg L^{-1} IAA). It is also noted that the C7 callus line produced low number of shoots per explant (1.8 - 11.3) when cultured into other regeneration media supplemented with BA and NAA. However, C8 callus line produced 44 shoots per explants when cultured into R3 regeneration medium. For the cultivar VFG 180 (3 -1), only 5 out of the 32 treatments were able to produce shoots (Figure 4B). The highest number of shoots per explant (51.3) was obtained from C6 callus line cultured into R4 medium while the lowest (6.5) was obtained from C3 callus line cultured into R3 medium. The cultivar Sids 40 showed the lowest regeneration efficiency among other cultivars. Only 1 treatment (C1 callus line cultured into R1 medium) produced 1.5 shoots per explant. All of the 8 callus lines for the wild type failed to regenerate shoots at all four regeneration media. The obtained results indicate that there were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. Such variations have been previously reported for garlic callus lines (Myers and Simon, 1998; Zheng et al., 2003). For example, garlic callus grown in medium with either auxins or cytokinins alone produced no shoots (Myers and Simon, 1999). Therefore, a combination of auxin and cytokinin is necessary for regeneration of garlic callus (Haque et al., 1997; Zheng et al., 2003; Khar et al., 2005), however, the type and concentration of PGRs were cultivar-dependent.

Adventitious shoot regeneration from shoot apices induced callus

All of the 8 callus lines induced from shoot apices were

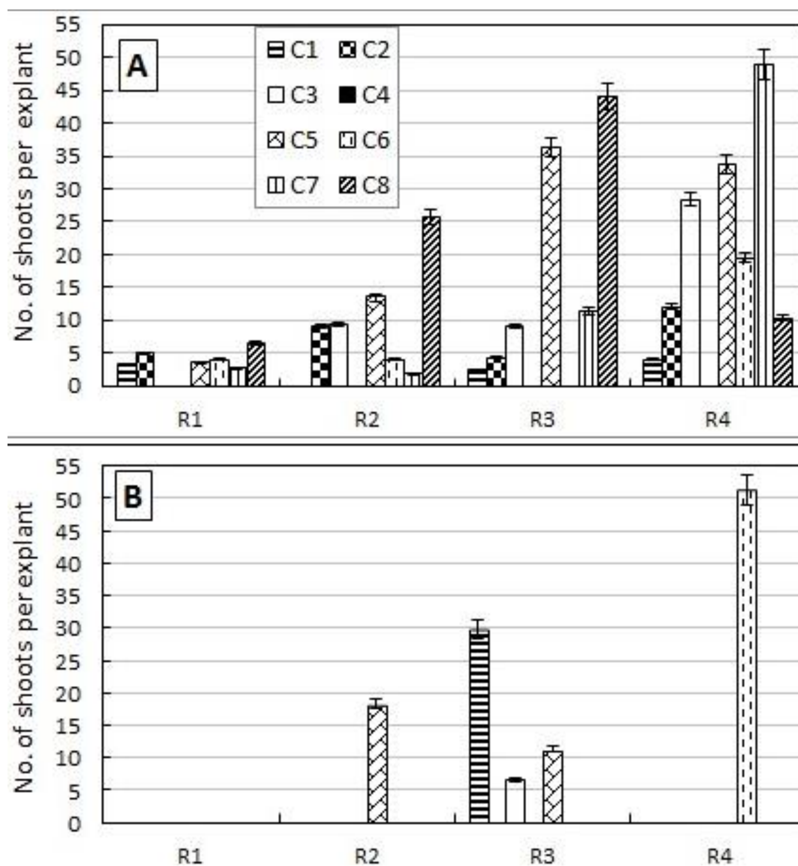

Figure 4. Adventitious shoot regeneration of eight callus lines induced from root apices of Balady cultivar (A) and VFG 180 (3 -1) (B). Regeneration media were supplemented with various PGRs: R1 (1.0 mg l^{-1} BA + 0.5 mg l^{-1}NAA); R2 (2.0 mg l^{-1}BA + 0.5 mg l^{-1}NAA); R3 (2.0 mg l^{-1}BA + 1.0 mg l^{-1}NAA); R4 (10.0 mg l^{-1}Kin + 2.0 mg l^{-1} IAA).

also transferred into four regeneration media rendering a group of 32 treatments for each cultivar as well as the wild type. The eight callus lines for both Sids 40 cultivar and the wild type failed to regenerate shoots. The cultivar VFG 180 (3 -1) showed very low regeneration efficiency. Only 1 treatment (C5 callus line cultured into R1 medium) produced 40.3 shoots per explant (data not shown). The Balady cultivar showed the highest regeneration efficiency among other cultivars. Thirty one out of 32 treatments regenerated shoots and the highest shoot number per explant (39) was obtained from C5 callus line cultured into R4 medium (Figure 5). Although shoot apices induced callus had higher regeneration efficiency than root apices induced callus but the number of regenerated shoots per explant was lower than that obtained from root apices induced callus. Six treatments for shoot apices induced callus lines produced more than 20 shoots per explant compared to one treatment for root apices induced callus lines (Figures 4A and 5).

In vitro cultures of garlic are greatly influenced by the genotype (Barandiaran et al., 1999a). It has been reported that using root tips as explant greatly increases the regeneration potential that can be achieved over other explant types such as shoot tips (Nagakubo et al., 1993; Mohamed-Yassen et al., 1994). Root tip explants are commonly used for the development of garlic regeneration system (Haque et al., 1997; Barandiaran et al., 1999b; Robledo-Paz et al., 2000; Keller and Senula, 2013).

Different genotypes of garlic also showed variations in time required for plant regeneration. It could be achieved within 4 and 9 months by Barandiaran et al. (1999a) and Myers and Simon (1998), respectively. In the present study, plant regeneration was obtained after 8 weeks in Balady cultivar and 12 months for Sids 40 and VFG 180 (3 - 1) cultivars while no regeneration could be obtained from the wild type.

In vitro rooting and acclimatization

The regenerated garlic shoots formed a well-developed root system within seven to eight days upon their culture on MS medium without PGRs (Figure 3C and D). Each

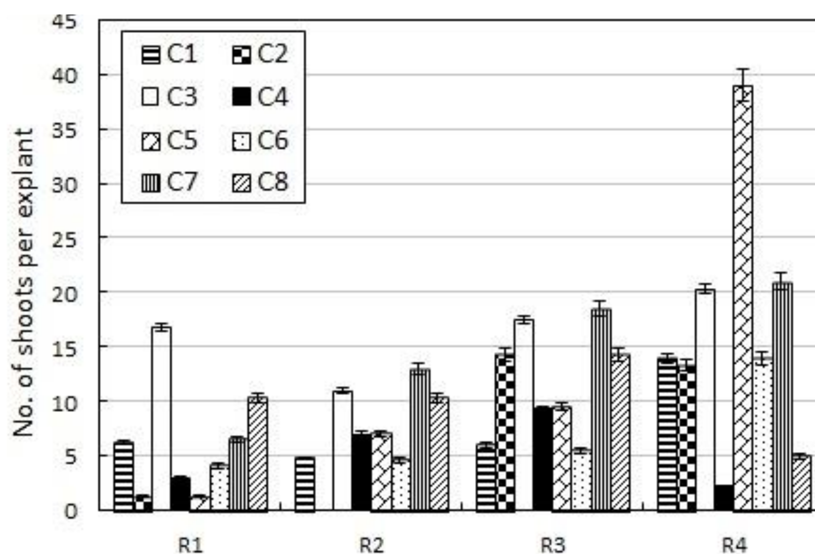

Figure 5. Adventitious shoot regeneration of eight callus lines induced from shoot apices of Balady cultivar and cultured into four regeneration media supplemented with various PGRs: R1 (1.0 mg l^{-1} BA + 0.5 mg l^{-1}NAA); R2 (2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA); R3 (2.0 mg l^{-1} BA + 1.0 mg l^{-1}NAA); R4 (10.0 mg l^{-1} Kin + 2.0 mg l^{-1} IAA).

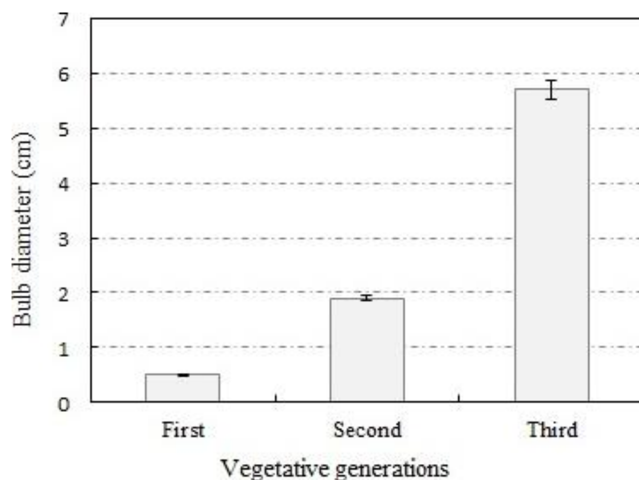

Figure 6. Development of bulb diameter during three successive vegetative generations of micropropagated garlic Balady cultivar.

plantlet had 2 - 5 roots with 2 - 3 cm in length. Previous reports indicated that *in vitro* rooting of garlic is easily achieved on MS medium without PGRs (Metwally and Zanata, 1996; Metwally et al., 2012; Keller and Senula, 2013). However, Tapia (1987) reported that garlic roots formed well on MS medium supplemented with Kin and IAA. Exposure of garlic plantlets to 50 µ mol m^{-2} s^{-1} PPF for two weeks permitted its acclimatization with 100% survival rate. It is well known that increasing light intensity reduces leaf length, length and width of cell and stomata index while increases leaf thickness (Rahim and Fordham, 1991). It has been reported that bulbs transferred to the soil sprouted in the first 3 months of culture with a 60% survival rate (Barandiaran et al., 1999a). At the end of the acclimatization stage, each plantlet gave a small and a non-divided bulb ranging from 0.2 - 1.2 cm in diameter (Figure 3E and F). The obtained plantlets differed in the shoot size; green color darkness, number of leaves and skin color of bulblet (white, light purple and purple). Such observations were also reported by Metwally and Zanata (1996) and Metwally et al. (2012). The size of the bulblets formed *in vitro* determines the quality of the plants developed in the field and the period of time required for commercial-sized cloves. In the present study, *in vitro* bulblets (0.5 cm in diameter) reached 1.9 and 5.7 cm in diameter in the second and the third vegetative generations, respectively (Figure 6). Thus, 3 years were required to obtain the commercial-sized cloves in Balady cultivar.

The present study investigates *in vitro* regeneration of three garlic cultivars that is Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type through adventitious shoot organogenesis. Shoot apices showed higher frequency of callus induction than root apices. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. There were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. plant regeneration was obtained after eight weeks in Balady cultivar and 12 months for Sids 40 and VFG 180 (3 - 1) cultivars while no regeneration could be obtained from the wild type. The Balady cultivar showed the highest regeneration efficiency with 39 shoots per explant.

Garlic plantlets of Balady cultivar derived through tissue culture required three vegetative generations to produce bulbs of commercial size.

Conflict of Interests

The authors declare no conflict of interests.

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Full Length Research Paper

In vivo cultivation technology and nutritional status of milky mushroom (*Calocybe indica*)

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The present study was conducted to evaluate the *in vivo* cultivation technology, proximate composition, mineral content and spectrum analysis of edible milky mushroom of *Calocybe indica*. Moisture, crude protein, carbohydrate, dietary fibre, total lipids, ash, ether extract, pH, nitrogen and carbon content in mushrooms were analysed. The results were found to be in 89, 14.9, 5.36, 8.02, 4.6, 7.05, 3.15, 5.4, 3.57 and 33.60% mg/100 g, respectively. The values of copper, manganese, zinc, iron, calcium, phosphorous, potassium and sodium content in mushrooms were found to be 0.44, 0.36, 0.05, 0.13, 0.51, 0.38, 1.35, 1.35 and 0.21 mg/100 g, respectively. Fourier transform infrared spectroscopy (FT-IR) spectrum of the mushroom indicated the presence of OH, COOH and NO_2 functional groups. The ultra-violet (UV) absorption showed at 294 nm with a shoulder at 321 and 379 nm indicating the presence of aromatic nature of the compounds. Data of this study suggests that mushrooms are rich in nutritional value.

Key words: *Calocybe indica*, Fourier transform infrared spectroscopy (FT-IR), UV Spectrometer, nutritional values.

INTRODUCTION

Mushrooms have been used as a part of regular diet for nutritional and medicinal values mostly by the ethnic group of Asian people from time immemorial. They contain minerals, vitamins and nutritive compounds, proteins, polysaccharide and have a low fat content (Khurshidul Zahid et al., 2004). The cultivation technology of mushroom is very simple, involves less cost and no special compost is needed. The main cultivation process of milky mushroom (*Calocybe indica*) is potentially new species to the world mushroom growers. It is a robust, fleshy, milky white, umbrella like mushroom, which resembles button mushroom. These species is suitable for hot humid climate and can be cultivated indoor in high temperature and high humidity areas. It grows well at a temperature range of 25-35°C and relative humidity more than 80%. It can be cultivated throughout the year in the entire plains of India and other countries. On an average, single mushroom weighs 55-60 g and mean yield is 356 g/bed, which accounts to 143% bio-efficiency. The milky mushroom have rich source of protein with content of 32.3% and fetches high market price compared to oyster mushrooms. It is highly suitable for drying, canning, soup powder preparation and pickle making. More than 80 edible mushrooms are considered for commercial

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exploitation, among these, milky mushroom has become the focal point of exploitation in India. This mushroom was first reported from India (Purkayastha et al., 1981). The Fourier-transform infrared spectroscopy is an analytical technique that enables the rapid, reagent less and high-throughput analysis of a diverse range of samples (Harrigan and Goodacre, 2003). Its importance lies in its ability to allow rapid and simultaneous characterization of different functional groups such as lipids, proteins, nucleic acids and polysaccharides in biological molecules and complex structures (Melin et al., 2004). Nutritional analysis of several mushroom species of different origins had been carried out in many laboratories in the world, but nutritional values of cultivated mushrooms remain speculative. Moreover, nutritional composition is affected by many factors; these include differences among strains, the composition of growth substrate, the method of cultivation, stage of harvesting and specific portion of the fruiting bodies used for analysis (Benjamin et al., 1995). Recently, *C. indica* have become an attractive functional food mainly because of their biochemical composition and antioxidant properties which have been reported to prevent oxidative damage by free radical and reactive oxygen species (ROS) and may prevent the occurrence of diseases like carcinogenesis, ageing, physical injury, infection and cardiovascular disease. Therefore, milky mushroom is considered as a better proxy for oyster mushroom notably in tropical regions with longer shelf life of three to four days. The present study was conducted to evaluate the *in vivo* cultivation technology and nutritional information of milky mushroom (*C. indica*).

MATERIALS AND METHODS

Collection of culture and maintenance

The pure culture of *C. indica* was obtained from Centre for Advanced study in Botany, University of Madras, Gundy Campus, and Chennai, India. The culture was maintained on potato dextrose agar slant and sub-cultured at regular monthly interval to sustain their fruiting vigour. They were preserved at 4°C temperature conditions. The above stock culture was used in further studies.

Spawn preparation

Wheat grain spawn of *C. indica* was prepared in glass bottles as described (Garcha et al., 1981). The wheat grain was semi boiled then 2% calcium carbonate and 0.2% gypsum was added to 1 kg of semi boiled wheat grain. The mother culture was prepared in glass bottles filling them ⅓ full and the working spawn was prepared in plastic bags capped with cotton plugs by rubber bands. The mother culture was grown on potato dextrose agar a medium that is first supplemented to the mother spawn and at full growth it was transferred to the working spawn bags of *C. indica.*

Preparation of bed and harvesting

The paddy straws were collected from local farmers of the Tuticorin district, Tamil Nadu, India. The straw was used as a substrate for cultivation. The substrate was soaked in cold water for 4 h. After draining excess water, the materials were treated in hot water (80°C) for 60 min and dried in shade. For the bed preparation, polythene bags of 60 x 30 cm size and 100 gauge thickness was used and cylindrical beds was prepared using 0.5 kg of substrate (dry weight) per bed. The filled paddy grain spawn of *C. indica* was used at 6% level to the wet weight of the substrate and the beds were spawned following layer method of spawning (Baskaran et al., 1978). After 10 to 15 days, when the beds were fully colonized by the mushroom fungus, they were cut into two equal halves and applied with casing soil to a height of 2 cm over the spawn run substrate in each of the half bed. The beds were uniformly and regularly sprayed with water until the last harvest. In total, three crops were harvested at intervals of three to five days.

Nutritional status of *C. indica*

The collected fresh mushrooms, shade dried and coarse powder was analysed for nutrients namely moisture, crude protein, fat, ash, crude fibre, minerals using FTIR and UV spectrometer.

Crude protein

Five grams of ground mushroom was taken with 50 ml 0.1 N NaOH and boiled for 30 min. The solution was cool to room temperature and centrifuged at 1000 rpm. The supernatant was collected and total protein content measured (Folch et al., 1957).

Crude lipid

The total lipid was determined by slight modified method (Lowry et al., 1951). The five grams of ground mushroom was suspended in 50 ml of chloroform: methanol (2:1 v/v) mixture then mixed thoroughly and let to stand for three days. The solution was filtered and centrifuged at 1000 *g* by a table centrifuge machine. The upper layer of methanol was removed by Pasteur pipette and chloroform was evaporated by heating. The remaining was the crude lipid.

Crude fiber

The total fibre was determined (Raghuramulu et al., 2003). Ten grams of moisture and fat-free sample was taken in a beaker and 200 ml of boiling 0.255 N H_2SO_4 was added. The mixture was boiled for 30 min keeping the volume constant by the addition of water at frequent intervals. The mixture was then filtered through a muslin cloth and the residue washed with hot water till free from acid. The material was then transferred to the same beaker, and 200 ml of boiling 0.313 N NaOH added. After boiling for 30 min, the mixture was filtered through a muslin cloth and the residue washed with hot water till free from alkali, followed by washing with some alcohol and ether. It was then transferred to a crucible, dried overnight at 80 to 100°C and weighed (We) in an electric balance. The crucible was heated in a muffle furnace at 600°C for 5-6 h, cooled and weighed again.

Total ash

One gram of the sample was weighed accurately into a crucible (Raghuramulu et al., 2003). The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 5 to 6 h at 600°C. It was then cooled in desiccators and weighed.

To ensure completion of ash, the crucible was then heated in the muffle furnace for 1 h, cooled and weighed. This was repeated till two consecutive weights were the same and the ash was almost white or greyish white in colour.

Mineral analysis

Total ash was taken for the analysis of mineral contents. Two millilitres of concentration HNO_3 was added to the ash and heated for 2 min. One drop of hydrogen peroxide was added into the solution. The solution was then transferred into a volumetric flask and total volume was made to 50 ml by adding deionised distilled water. This was then used to analyze the contents of calcium (Ca), iron (Fe), manganese (Mn), magnesium (mg), zinc (Zn), selenium (Se) and arsenic (As) by flame and graphite method with atomic absorption spectrophotometer (Perkin Elmer: AS 80).

Fourier transform infrared spectroscopy (FT-IR) spectral analysis

The lyophilized samples of *C. indica* (10 mg) were mixed with 100 mg of dried potassium bromide (kBr) and compressed to prepare a salt disc. The disc was then read spectrophotometrically (FTIR-8400S, SHIMADZU, Japan). The frequencies of different components present in each sample were analyzed (Line et al., 2008).

UV spectrophotometer

UV spectrum for the test powder was recorded in SHIMADZU-UVVIS-160 spectrophotometer, Japan. 1 mg of sample was dissolved in 3 ml of high performance liquid chromatography (HPLC) grade methanol and the mix was recorded between 200 and 500 nm where the extracts exhibited absorption maxima.

RESULTS

Figure 1 shows *C. indica* grown on paddy straw and were analysed for nutrients such as moisture: moisture content was found to be 8.9%. The hundred grams of dry *C. indica* contains of moisture, proteins, carbohydrate, fibre, fat, ash, ether extract, pH, total nitrogen and total carbon were found to be 89, 14.09, 13.09, 5.63, 8.02, 4.6, 7.05, 3.57, 33.60 and 5.4 mg/100 g, respectively (Table 1). The minerals content of copper, manganese, zinc, iron, calcium, phosphorous, potassium and sodium were found to be 0.44, 0.036, 0.05, 0.13, 0.51, 0.38, 1.35 and 0.21, respectively (Table 2).

Spectrum analysis

FT-IR vibrational frequencies obtained at 3315 cm^{-1} inferred the O-H stretching (hydrogen bonded intermolecular), the bands of at 2490 cm^{-1}, the bands at 2242 and 2074 cm^{-1} corresponded to N-O bending, C=O stretching at 1870 cm^{-1}, C=O symmetric carboxylate stretching at 1413 cm^{-1}. The C-O stretching appeared at 1116 cm^{-1}. O-C=C asymmetric stretching was at 1150 cm^{-1} and a sharp C-X out of plane bending at 603 cm^{-1}. All these characteristic bands of the FT-IR spectrum of mushroom indicated the presence of OH, COOH and NO_2 groups (Figure 3). The UV absorption maxima appeared at 294 nm with a shoulder at 321 and 379 nm indicating the aromatic nature of the compounds (Figure 2).

DISCUSSION

Mushrooms are generally classified into four groups: edible mushrooms, medicinal mushrooms, poisonous mushrooms and magic or hallucinogenic mushrooms. Edible mushrooms are ideal healthy foods. They may contribute enormously to the supply of both macro and micro nutrients in our diet. They are considered to be the potential source of carbohydrates, proteins, fat, and minerals. All of which contribute to the food value. Cultivated *C. indica* using 49 different substrates including various plant products, crop residues and leaves recorded the higher yield from paddy straw supplemented with 5% maize meal (Mahesh and Yadav, 2006). In the present study, paddy straw was used for the cultivation of *C. indica*. The crude protein, fat and total carbohydrate contents of *C. indica* and *P. sajor - caju* analysed at various growth stages exhibited strikingly different results (Sivapraksam and Ramaraj, 1997). In the present study, protein, carbohydrate, amino acids and lipid contents of *C. Indica* was analyzed (Table 2).The total fat content was greater in *C. indica* which is significant to *P. sajor-caju* and *P. florida.* It was also significantly richer in carbohydrates than the three species of *Pleurotus* (Nuhu et al., 2008). On the other hand the fiber content in *C. indica* is significantly lower about 8% than that in *Pleurotus* spp. Mushroom are also rich in mineral contents. The total ash content found was 7.5. In the present study, the FTIR spectrum of mushroom indicated the presence of OH, COOH and NO_2 groups. Similarly in the previous study of FT-IR spectra of *Pleurotus* spp. straw powder, a broad stretching band was observed at 3416 $cm^{-}1$ due to the presence of OH and NH groups (Ranjani et al., 2013). The biosynthesis of *C. indica* extract was shown at UV 200 to 600 nm which was observed in the presence of silver nanoparticles (Sujath et al., 2013) similar to the present study of UV absorption maxima which appeared at 294 nm with a shoulder at 321 and 379 nm indicating the aromatic nature of the compounds. In conclusion, the chemical compositions of edible mushrooms determine their nutritional value and sensory properties as also mentioned (Shah et al., 1997; Manzi et al., 2001). These data suggest that dietary *C. indica* is a good source of nutrients specially protein and fibre. Mushrooms are rich in protein, edible fibre and minerals but lipid content is low. These results also indicate that the studied mushrooms have good nutritive value for human.

Figure 1. Cultivation technology of *Calocybe indica.*

Table 1. Proximate analysis of *Calocybe indica.*

Nutritional parameters g/100 g	Value (%)
Moisture	89
Crude protein	14.09
Carbohydrate	5.63
Crude fibre	8.02
Lipid	4.6
Ash	7.05
Ether extract	3.15
(pH) 5% solution	5.4
Total nitrogen	3.57
Organic carbon	33.60

Table 2. Micro nutrition analysis of *Calocybe indica.*

Micro nutrient analysis	Value (mg/g)
Copper	0.44
Manganese	0.36
Zinc	0.05
Iron	0.13
Calcium	0.51
Phosphorous	0.38
Potassium	1.35
Sodium	0.21

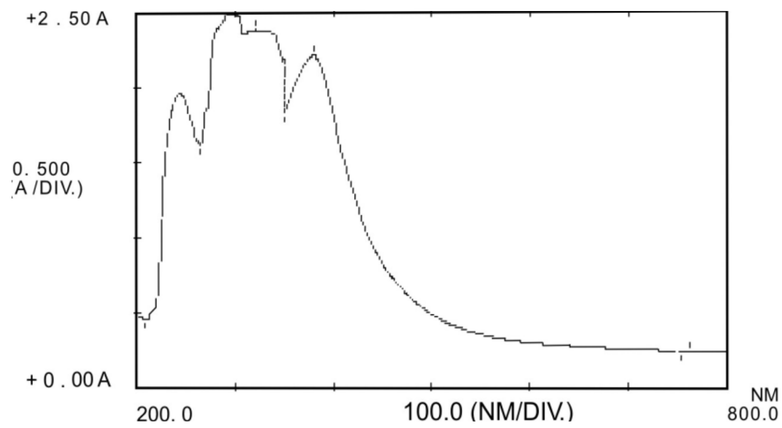

Figure 2. UV spectrum of *Calocybe indica.*

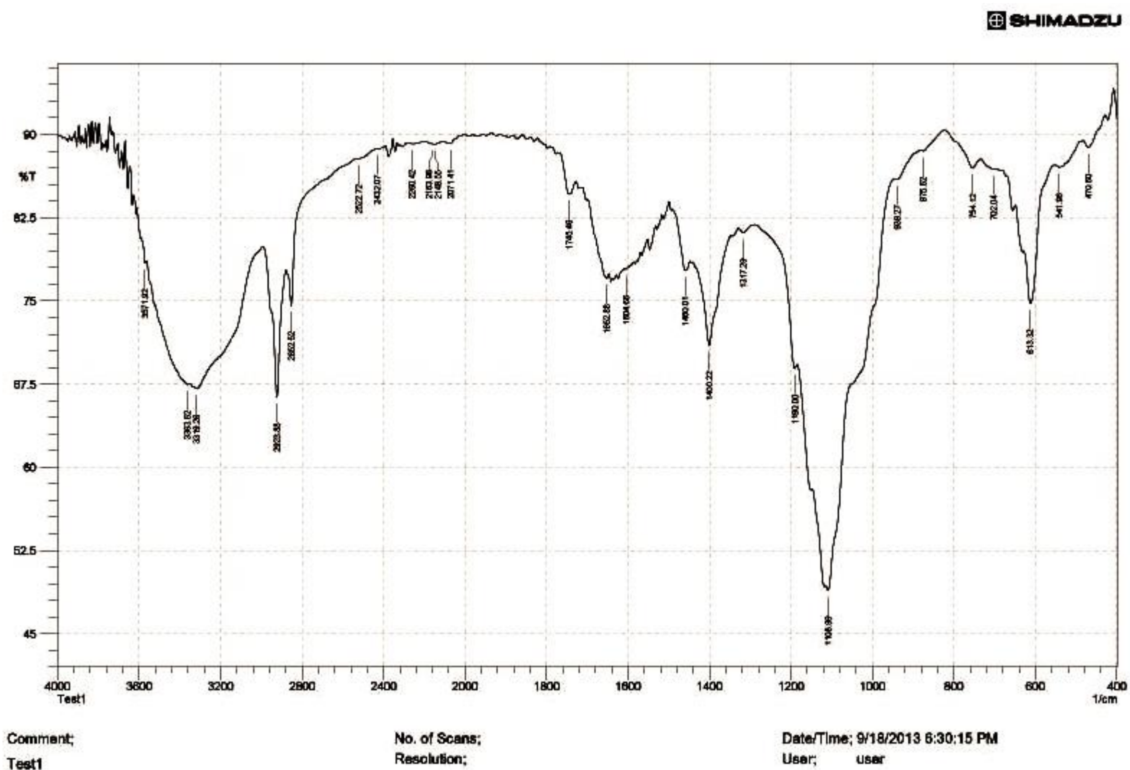

Figure 3. FT-IR Spectrum of *Calocybe indica.*

Conflict of Interests

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

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Full Length Research Paper

Evaluation of the quality attributes of wheat composite (wheat-cassava, wheat-plantain and wheat-rice) flours in bread making

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Composite flour was produced with wheat and other crops like rice, plantain and cassava at 20% substitution. The flour mixes were evaluated for proximate, physico-chemical properties and sensory evaluation was carried out on bread samples produced from these mixes. The moisture contents of these flours ranged from 11.00 to 12.30%. Also, ash and crude fibre contents ranged from 1.03 to 1.50% and 0.70 to 1.17%, respectively. The protein content ranged between 9.30 - 10.90% with wheat- cassava flour mixes having the lowest while the sample with 100% wheat had the highest protein content. The fat content ranged from 1.50 to 1.93%. Water absorption capacity and pasting viscosity of the flours ranged from 59.50 to 66.60% and 466 to 893 RVU, respectively. The bulk density of the samples ranged between 0.48 and 0.88 g/ml while pH values ranged from 6.57 to 6.70. The sensory analysis reflected that bread produced from 100% wheat flour was much accepted by the panelists but the ratings were close indicating a probability at 20% substitution.

Key words: Bread, composite flour, proximate composition, physico-chemical properties, sensory evaluation.

INTRODUCTION

Bread is an important staple food in both developed and developing countries and it has become the second most widely consumed non-indigenous food products after rice in Nigeria (Shittu et al., 2007). Wheat (*Triticum aestivum* Desf.) flour has been the major ingredient of bread for many years because of its functional proteins (Abdelghafor et al., 2011). Wheat gluten imparts to dough physical properties that differ from those of dough made from other cereal grains. It is gluten formation, rather than any distinctive nutritive property, that gives wheat its prominence in the bread making. Wheat produces white flour having a unique property of wheat proteins, which can produce dough having the strength and elasticity required to produce low-density bread, biscuit and pastries of desirable texture and flour which makes wheat the most popular cereal grain known in the world (Ihekoronye and Ngoddy, 1985).

However, wheat is not suitable for cultivation in the tropical areas for climatic reasons (Edema et al., 2005). In an effort to help the third world countries reduce or stabilize their importation, the FAO in 1957 started a study on the technological feasibility of the use of

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composite flours for the production of biscuits, bread and pastry products (Aliyu and Sani, 2009). Several developing countries have encouraged the initiation of programs to evaluate the feasibility of alternative locally available flours as a substitute for wheat flour. Many efforts have been carried out to promote the use of composite flours, in which a portion of wheat flour is replaced by locally grown crops, to be used in bread, thereby decreasing the cost associated with imported wheat (Olaoye et al., 2006).

Plantain is a popular dietary staple due to its versatility and good nutritional value. It is starchy, less sweet variety of banana that can be used either ripe or unripe; they are invaluable source of carbohydrate, comparable in nutritive value to yam or potato and are useful as a variant on the usual staple foods (Ukhum and Ukpebor, 1991). The fruit is an excellent source of nutrient; it contains about 32% carbohydrate, 1% protein, 0.02% fat, some vitamins and mineral contents (Kure et al., 1998). It is recommended to produce plantain flour from green fruits since it has high starch content of about 35% on wet basis (Olaoye et al., 2006).

Rice (*Oryza sativa)* is one of the most important cereals of the world. It is produced locally in most part of Nigeria, namely, Abakaliki, Bida, Abeokuta, Mokwa, among others, where it is used mainly for human consumption and it is referred to as *ofada* rice. Estimated annual production of rice in Nigeria is about 3 million tonnes (Adebowale et al., 2010). *Ofada* rice is the mostly cultivated rice in Nigeria especially in the Southwestern agro ecological zone. Oko and Ugwu (2011) reported that the local rice varieties in Nigeria contained carbohydrates ranging from 76.92 to 86.03%.

Some previous studies have been conducted on the use of composite flour for bread making purposes; Olaoye et al. (2006), produced bread from wheat, plantain and soy beans; Anjum et al. (2008) produced bread from wheat-potato composite flour; Shittu et al. (2007) produced bread from cassava-wheat flour; Abdelghafor et al. (2011) produced bread from wheat and sorghum and others are Dhingra and Jood (2004), Hsu et al. (2004), Khalil et al. (2000), McWatter et al. (2004).

The limited production of wheat in the tropics due to climatic factors, as well as the prohibitive cost of importation and ever increasing demand for baked products have necessitated supplements or substitutes. The Federal Government of Nigeria has mandated the use of composite cassava-wheat flour for baking by adding a minimum of 10% cassava flour to wheat for a start.

This study, however evaluated the probability of using a local rice variety, plantain and cassava flour which are crops that are locally produced in Nigeria and have been used and reported as having probable use as composite flour in making of bread (Mepba et al., 2007; Oluwamukomi et al., 2011) above the recommended substitution level and to determine the probable uses at higher substitution rates.

This work aimed to determine the proximate composition; physico-chemical properties of the mixes; and to evaluate the consumer acceptability of bread produced from wheat composite flours (wheat-cassava, wheat-plantain and wheat- rice).

MATERIALS AND METHODS

Processed unfermented cassava flour was obtained from Federal Institute of Industrial Research, Oshodi (FIRRO) in Lagos. A local variety of rice (*ofada*) and bunch of plantains were purchased from a local market in Ogbomoso, Oyo State, Nigeria. Wheat flour, yeast, sugar, fat and salt were obtained from the Quality Control Bakery of Flour Mills of Nigeria Plc., Apapa, Lagos, Nigeria.

Preparation of plantain and rice flours

Unripe plantains were sorted and graded for quality. The plantains were washed, peeled and thinly sliced into about 2 cm thickness using the manual plantain slices. The sliced plantains were immersed in 0.03% of sodium metabisulphite solution for 10 min to obtain white flour dried in the cabinet dryer at 60°C for 24 h. They were drained out of the solution and dried. The dried plantain slices were milled into flour using a hammer mill and sieved through 250 µm aperture sieve. The flour was packed and sealed in polyethylene bags until analysis.

Rice grains were cleaned, conditioned for 5 min and then sun dried. The dried grains were milled using the plate disc mill. The flour obtained was cooled, sieved and packed in polyethylene bags until analysis.

Formulation of composite flours

Composite flours in this study were formulated by blending wheat flour with non-wheat (cassava, plantain and rice) flours in the ratio 80:20 using the Buhler flour blender to obtain homogenous wheat-cassava, wheat-plantain and wheat-rice flours.

Production of bread

Bread was produced with bulk fermentation, according to AACC (1984) which employs a bulk fermentation process. Five hundred gram of each of the composite flour sample was weighed along with the required amount of water and other ingredients; sugar (6 g), fat (5 g), yeast (2.5 g), salt (1.5 g) and water (0.5 L) to obtain dough, which was kneaded on a pastry-board to smoothen it. The dough was initially fermented for 2 h at 30°C before being subsequently knocked back by kneading to expel carbon dioxide and tighten-up the dough to improve the texture of the final product. The secondary fermentation also lasted for 2 h at 30°C. The dough were then sized and molded into the baking pans for final proving at 30°C for 2 h. Dough baking was carried out in the oven at a temperature of 230°C for 25 min. These ingredients of the same quantity were also added to the 100% wheat flour as control.

Proximate analysis

The moisture, ash, crude protein, fat, crude fibre were determined as described by AOAC methods (AOAC, 1990) while the carbohydrate was calculated by difference. Moisture content (%

Table 1. Proximate composition of flours.

Sample code	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Crude fibre (%)	Carbohydrate (%)
A	11.00^{c}	10.90^{a}	1.80^{a}	1.17^{b}	0.70^{c}	75.13^{c}
B	12.00^{b}	9.30^{b}	1.50^{b}	1.03^{c}	0.90^{b}	76.17^{a}
C	12.30^{a}	9.43^{b}	1.93^{a}	1.50^{a}	1.03^{ab}	74.84^{d}
D	12.10^{b}	9.50^{b}	1.63^{b}	1.43^{a}	1.17^{a}	75.34^{b}

Means with the same alphabet in the same column are not significantly different. ($p< 0.05$). A, 100% wheat flour; B, 80% wheat flour and 20% cassava flour; C, 80% wheat flour and 20% plantain flour; and D, 80% wheat flour and 20% rice flour.

MC) was determined by drying samples in an oven at 105°C for 16 h. Crude protein percentage (% CP) was determined by Kjeldahl method and the percentage nitrogen obtained was used to calculate the% CP using the relationship:% CP =% N × 6.25. Fat content (%) was determined using Soxhlet extraction technique and percentage ash (%) was determined by incinerating the samples in a muffle furnace at 600°C for 6 h. The ash was cooled in a desiccator and weighed. Crude fibre percentage (% CF) was determined by dilute acid and alkali hydrolysis and the carbohydrate was determined by difference that is,

% Carbohydrate = 100 - (moisture + ash + fat + protein + fiber)

Determination of physico-chemical properties

Water absorption capacity (WAC) was determined using the method of Mbofung et al. (2006). One gram of the flour was mixed with 10 ml of water in a centrifuge tube and allowed to stand at room temperature (30 ± 2°C) for 1 h. It was then centrifuged at 3000 × *g* for 10 min. The volume of water on the sediment water measured. Water absorption capacities were calculated as ml of water absorbed per gram of flour. The bulk density of the flour samples were determined by the method of Akpapunam and Markakis (1981). A 50 g flour sample was put into a 100 ml measuring cylinder and tapped to a constant volume. The bulk density (g/ml) was calculated as weight of flour (g) divided by flour volume (ml). Pasting characteristics were determined with a Rapid Visco Analyzer (RVA) (ModelRVA 3D+, Newport Scientific Australia) as described by Ikegwu et al. (2009). The pH was determined using a pH meter (cheaker 3 model).

Sensory evaluation

Sensory evaluation of the bread samples were conducted after baking by a 25-member panel, randomly selected from students of Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria. Panelists evaluated the samples for crust colour, crumb colour, flavour, texture, sweetness and general acceptability using a product-oriented test. Nine point hedonic scale ranging from 9 = like extremely to 1 = dislike extremely was used by the panelists.

Data analysis

Statistical analysis of all data was done with the statistical analysis systems (SAS) package (version 9.2 of SAS institute Inc, 2003). The statistical analysis was carried out using analysis of variance (ANOVA); significant differences ($p< 0.05$) in all data were determined by general linear model procedure (GLM) while least significant difference (LSD) was used to separate the means.

RESULTS AND DISCUSSION

Proximate analysis

The data obtained are as shown in Table 1. The moisture content of the flour samples ranged from 11.00 to 12.30% (dry basis). The sample with 20% plantain flour substitution had the highest moisture content and the control which is 100% wheat flour had the lowest. There was no significant ($p<0.05$) difference between the moisture content of the samples substituted with 20% cassava flour and 20% rice flour. The protein contents were found to be between the range of 9.00 and 10.90%. The sample with 100% wheat had the highest protein content and it was significantly ($p<0.05$) different from other samples. This is probably a reflection of gluten content in wheat flour. This value is close to the value obtained by Mepba et al. (2007), 12.86% and Ugwuona (2009) 12.93% for 100% wheat flour. The protein decreased with composite flour substitution probably due to low protein contents of the substituted flour and the decrease was in line with the protein values of these flours. Rice flour had the highest protein content among the composite flour, followed by plantain flour while the least protein value was recorded with cassava flour. The fat content of the samples ranged from 1.50 to 1.93% where sample with plantain flour substitution had the highest and the sample with 20% cassava flour had the lowest fat content. This is also a reflection of the fat components of the composite flours, the order of increase or decrease was based on the fat contents of the composite flours. The mineral contents of the flour mixes ranged between 1.03 to 1.50%. There was no significant ($p<0.05$) difference between the sample with plantain substitution and that of rice flour substitution. This is a reflection of the mineral contents of the flours. The crude fibre ranged between 0.70 and 1.17% and all the samples are significantly ($p<0.05$) different from each other. The sample with 20% rice flour had the highest crude fibre while 100% wheat flour had the lowest. The carbohydrate content ranged from 74.84 to 76.17% and are significantly ($p<0.05$) different from each other. Highest value was recorded with sample that had 20% cassava flour substitution while the sample with 20% plantain flour substitution had the lowest value.

Table 2. Physico-chemical analysis of flour.

Sample	Water absorption capacity (%)	Bulk density (g/cm^3)	Pasting viscosity (RVU)	pH	Baking time (minutes)
A	60.90^{c}	0.88^{a}	626^{b}	6.57^{c}	24^{a}
B	66.60^{a}	0.48^{b}	518^{c}	6.70^{a}	24^{a}
C	59.50^{d}	0.57^{b}	893^{a}	6.67^{b}	24^{a}
D	66.00^{b}	0.73^{ab}	466^{d}	6.58^{c}	24^{a}

Means with the same alphabet in the same row are not significantly different. (p< 0.05).). A, 100% wheat flour; B, 80% wheat flour and 20% cassava flour; C, 80% wheat flour and 20% plantain flour; and D, 80% wheat flour and 20% rice flour.

Table 3. Taste panel score of wheat and composite flours bread.

Sample	Crust colour	Crumb colour	Flavour	Texture	Sweetness	General acceptability
A	3.6_{b}	8.6_{a}	8.4_{a}	8.4_{a}	7.4_{a}	6.8_{a}
B	4.2_{b}	3.3_{b}	7.2_{b}	7.5_{b}	6.3_{b}	5.4_{b}
C	7.2_{a}	3.0_{b}	5.1_{c}	6.0_{c}	4.7_{c}	4.5_{c}
D	3.9_{b}	3.5_{b}	7.8_{b}	5.4_{c}	6.8_{a}	6.1_{b}

Mean values in the same column with different subscripts differ significantly.). A, 100% wheat flour; B, 80% wheat flour and 20% cassava flour; C, 80% wheat flour and 20% plantain flour; and D, 80% wheat flour and 20% rice flour.

Physico-chemical properties of flours

The results of physico-chemical properties are as shown in Table 2. The water absorption capacity of the flour samples ranged from 59.50 to 66.60% and the values are significantly ($p<0.05$) different. The sample with 20% cassava flour substitution had the highest water absorption capacity, followed by that of rice, 100% wheat while the sample with plantain flour substitution had the lowest. This is also a reflection of the protein and carbohydrate contents of these flours. Water absorption is the amount of water absorbed by the flour to produce dough of workable consistency. It is determined by the protein content of the flour, the amount of starch damaged during milling and the presence of non-starch carbohydrates. It is desirable that flours for bread-making possess a high water absorption capacity at normal working consistencies so that the yield of dough, and hence bread, will be relatively high (Ma et al., 2007).

The values obtained for bulk density of the flours ranged from 0.48 to 0.88 g/cm^3, where the sample with 100% wheat had the highest and the sample with cassava flour substitution recorded the lowest. There were significant ($p<0.05$) differences among the samples except the sample with 20% cassava flour substitution and 20% plantain substitution. The bulk density is generally affected by the particle size and the density of the flour and it is very important in determining the packaging requirement, material handling and application in wet processing in the food industry (Adebowale et al., 2008) indicating a lesser package requirement with 20% cassava flour substitution.

The pasting viscosity of the flours ranged between 466 and 893 RVU. The pH values ranged from 6.58 to 6.70 and the sample with cassava flour substitution had the highest value.

Sensory evaluation

The crumb colour of 100% wheat and that of 20% cassava flour substitution was not significantly different. The crust colour of the flours substituted and the 100% wheat flour were not significantly different while the flavour, texture, sweetness and general acceptability were significantly ($p<0.05$) different as presented in Table 3.

Conclusion

Though some of the quality attributes of bread were significantly ($p<0.05$) different from 100% wheat flour substitution as expected, the ratings were not really far from each other indicating that some of these attributes could be worked on and thus making substitution at 20% of these composite flours possible.

Conflict of Interests

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

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Full Length Research Paper

Optimization of extraction process for ustiloxins A and B from rice false smut balls using an orthogonal array design

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Optimization of four factors, namely methanol concentration (*A*), extraction pH value (*B*), material-to-solvent ratio (*C*) and extraction times (*D*), for extraction of ustiloxins A and B from rice false smut balls was achieved by using an L_{18} orthogonal array design with three levels and four factors. The results show that the optimum conditions for ustiloxins A and B extraction should be $A_1B_2C_2D_3$ corresponding to methanol concentration at 10%, extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL) and extraction times as 3, respectively. Under the optimum extraction condition, the content of ustiloxins A and B in rice false smut balls was analyzed to be 0.80 and 0.57 mg/g, respectively, on a dry weight basis.

Key words: Rice false smut balls, *Villosiclava virens*, *Ustilaginoidea virens*, ustiloxins A and B, orthogonal array design, extraction optimization.

INTRODUCTION

The false smut of rice (*Oryza sativa* L.) is an emerging, increasingly significant and worldwide fungal disease (Brooks et al., 2009; Ashizawa et al., 2010; Ladhalakshmi et al., 2012; Tang et al., 2013). Its pathogen *Villosiclava virens* (Nakata) Tanaka and Tanaka (anamorph: *Ustilaginoidea virens* Takahashi) can produce ustiloxins which are cyclopeptide mycotoxins containing a 13-membered cyclic core structure (Koiso et al., 1994; Tanaka et al., 2008; Zhou et al., 2012). Ustiloxin A as well as the crude water extract of rice false smut balls were reported to cause liver and kidney damage in mice (Nakamura et al., 1994). This indicates that the false smut balls as well as false smut pathogen-infected rice food and forage create concerns for food and feed safety. Furthermore, ustiloxins are toxic to plants and animals especially with their antimitotic activity by inhibiting

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Table 1. Factors and levels of the orthogonal test.

Factor	Level 1	Level 2	Level 3
Methanol concentration (%, v/v)	10	30	70
Extraction pH value	2	6	10
Material-to-solvent ratio (g/mL)	1:20	1:30	1:40
Extraction times	1	2	3

microtubule assembly and cell skeleton formation (Luduena et al., 1994). As tubulin is an established chemotherapeutic target, mitotic arrest has been considered to be important in cancer chemotherapy. Therefore, ustiloxins provide an attractive entry to the study of antimitotic natural products and their biochemical effects at the molecular and cellular levels on the target. Ustiloxins have been regarded as a novel resource with their potential applications for medicinal and agro-chemical purposes, acting as anticancer and anthelmintic agents (Li et al., 1995, 2006, 2008; Zhou et al., 2012).

Of five elucidated ustiloxins, both ustiloxins A and B are more structurally complicated and have been approved to be more effective to show cytotoxic activity than other ustiloxins (that is,, ustiloxins C, D and F) (Koiso et al., 1994). Both ustiloxins A and B were also the predominant toxin components in the rice false smut balls which were composed of the chlamydospores and mycelia of the pathogen (Shan et al., 2012). As ustiloxins A and B have not been successfully synthesized under *in vitro* till now (Zhou et al., 2012), rice false smut balls and cultured mycelia have been regarded as the only sources of ustiloxins A and B.

In order to speed up investigation and application of ustiloxins, one of the most important approaches is to efficiently obtain ustiloxins. To the best of our knowledge, it has not yet been reported in detail regarding the extraction process of ustiloxins A and B from rice false smut balls or cultured mycelia. The purpose of this investigation was to seek a practical method for ustiloxin extraction from the samples (that is false smut balls, cultured mycelia, grains, forage rice and their products). In this study, four factors, namely methanol concentration (that is, 10, 30 and 70%, v/v), extraction pH value (that is, 2, 6 and 10), material-to-solvent ratio (that is 1:20, 1:30 and 1:40, g/mL), and extraction times (that is, 1, 2 and 3) along with their three levels, for extraction of ustiloxins A and B from rice false smut balls were optimized by using the L_{18} orthogonal array design (OAD) based on the single-factor test. Verification experiment for the corresponding factors under the optimum condition was also carried out.

MATERIALS AND METHODS

Materials

The rice false smut balls, which were mainly composed of the chlamydospores and mycelia of the rice false smut pathogen (*Villosiclava virens*), were collected from the southwestern part of Shandong Province of China during cropping season. The materials were left to dry in shade at room temperature (23 ± 2°C) to a constant weight, and were then stored in the sterilized sealed plastic bags at -20°C until required.

Extraction procedure

The first step of the experimental design is to determine the important factors, whose variation has a critical effect on the extraction of ustiloxins A and B from rice false smut balls. 200 mg of rice false smut balls was powered by using pestle and motor, and was put into a 35-mL extraction tube under different extraction conditions. The basal extraction condition was at pH 6.0 and 60°C with extraction time as 30 min, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 2. Methanol concentration (10-100%), extraction temperature (20-100°C), extraction pH value (2-10), extraction time (10-50 min), material-to-solvent ratio (1:10-1:50, g/mL), and extraction times (1-5) were evaluated for the extraction of ustiloxins A and B from rice false smut balls. The crude extract solution was filtered through Whatman no. 1 filter paper. The solvents were then removed by using a rotary evaporator at 50°C.

Orthogonal test

To further optimize the ustiloxin extraction from rice false smut balls, four parameters (methanol concentration, extraction pH value, material-to-solvent ratio, and extraction times) were used as the factors of the orthogonal experiment (Zhang et al., 2009; Guo et al., 2012; Khongsay et al., 2012). The selected factors and their levels in the experiment are shown in Table 1. The L_{18} (3^7) orthogonal design is shown in Table 2. The data were analyzed using the orthogonality experiment assistant software II V3.1 (Sharetop Software Studio, China).

HPLC analysis of ustiloxins A and B

Quantitative analysis of ustiloxins A and B by HPLC was carried out as previously described (Shan et al., 2012). The rice false smut balls sample was prepared with the optimum condition mentioned above. The concentrated extract was dissolved in 2 mL of methanol-water (15:85, v/v) and filtered through a filter (pore size, 0.22 μm) before analysis. Ustiloxin content was analyzed by a Prominence LC-20A high-performance liquid chromatography (HPLC) system (Shimadzu, Japan), which consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, and a CBM-20Alite system controller. Chromatographic separations were performed at 30°C using Synergi reversed-phase hydro-C_{18} column (250 mm x 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA). The mobile phase, composed of methanol with water containing 0.02% trifluoacetic acid (TFA) (15:85, v/v); was eluted at a flow rate of 1.0 mL/min, with

Table 2. The L_{18} orthogonal design.

Run	Methanol Concentration (%, *A*)	Extraction pH value (*B*)	Material-to-solvent ratio (g/mL, *C*)	Extraction times (*D*)	Blank 1 (*E*)	Blank 2 (*F*)	Blank 3 (*G*)
1	0	2	1:20	1	1	1	1
2	0	6	1:30	2	2	2	2
3	0	10	1:40	3	3	3	3
4	30	2	1:20	2	2	3	3
5	30	6	1:30	3	3	1	1
6	30	10	1:40	1	1	2	2
7	70	2	1:30	1	3	2	3
8	70	6	1:40	2	1	3	1
9	70	10	1:20	3	2	1	2
10	0	2	1:40	3	2	2	1
11	0	6	1:20	1	3	3	2
12	0	10	1:30	2	1	1	3
13	30	2	1:30	3	1	3	2
14	30	6	1:40	1	2	1	3
15	30	10	1:20	2	3	2	1
16	70	2	1:40	2	3	1	2
17	70	6	1:20	3	1	2	3
18	70	10	1:30	1	2	3	1

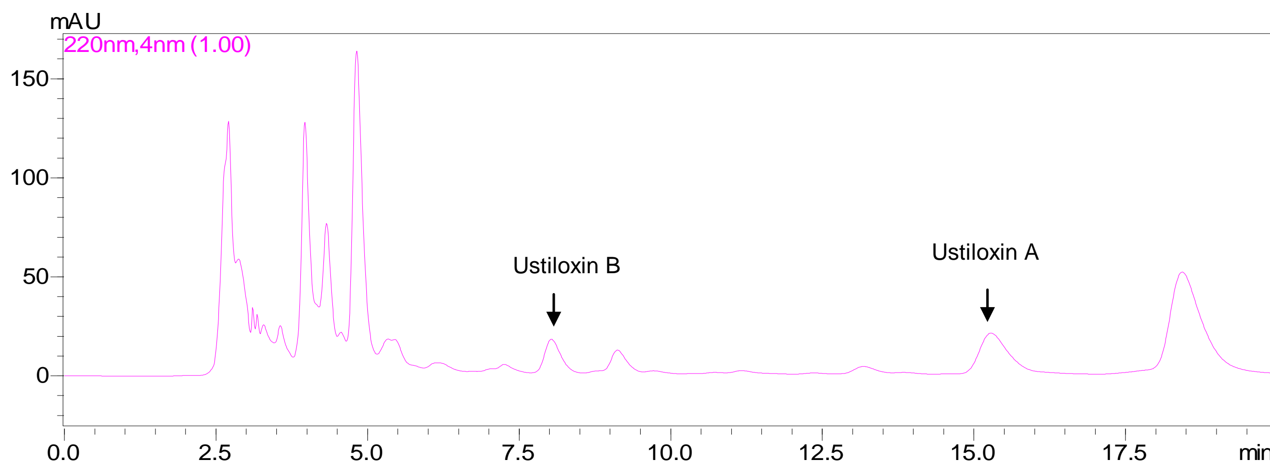

Figure 1. HPLC chromatogram of ustiloxins A and B in the extract of rice false smut balls.

UV detection at 220 nm and a total analysis time of 20 min. The LC solution multi-PDA workstation was employed to acquire and process chromatographic data. The HPLC chromatogram of ustiloxins A and B in the extract of rice false smut balls is shown in Figure 1.

Statistical analysis

All experiments were carried out in triplicate, and the results were represented by their mean values and the standard deviations (SD). Analysis of variance (ANOVA) was used in a completely random design. Duncan's multiple range test and Pearson's correlation coefficients were used to compare the data. The data were submitted to analysis of variance to detect significant differences by PROC ANOVA of SAS version 8.2.

RESULTS AND DISCUSSION

Single-factor test on extraction of ustiloxins A and B

Effects of each factor along with its five levels on

Table 3. Effects of different factors on extraction of ustiloxins A and B.

Factor and its levels		Content of ustiloxin A (mg/g)	Content of ustiloxin B (mg/g)
Methanol concentration (%, v/v)	10	0.71 ± 0.03[a]	0.61 ± 0.02[a]
	30	0.67 ± 0.01[b]	0.55 ± 0.01[b]
	50	0.58 ± 0.01[c]	0.46 ± 0.01[c]
	70	0.56 ± 0.00[c]	0.42 ± 0.01[d]
	100	0.40 ± 0.00[d]	0.30 ± 0.01[e]
Extraction temperature (°C)	20	0.50 ± 0.03[a]	0.41 ± 0.02[a]
	40	0.49 ± 0.01[a]	0.41 ± 0.01[a]
	60	0.49 ± 0.00[a]	0.41 ± 0.00[a]
	80	0.47 ± 0.01[a]	0.37 ± 0.02[b]
	100	0.19 ± 0.02[b]	0.16 ± 0.02[c]
Extraction pH value	2	0.49 ± 0.01[b]	0.36 ± 0.01[b]
	4	0.54 ± 0.02[ab]	0.42 ± 0.01[a]
	6	0.56 ± 0.01[a]	0.43 ± 0.01[a]
	8	0.55 ± 0.01[a]	0.43 ± 0.01[a]
	10	0.44 ± 0.03[c]	0.31 ± 0.01[c]
Extraction time (min)	10	0.52 ± 0.00[a]	0.39 ± 0.00[a]
	20	0.52 ± 0.01[a]	0.37 ± 0.03[a]
	30	0.52 ± 0.00[a]	0.39 ± 0.00[a]
	40	0.51 ± 0.04[a]	0.38 ± 0.01[a]
	50	0.50 ± 0.02[a]	0.36 ± 0.01[a]
Material-to-solvent ratio (g/mL)	1:10	0.15 ± 0.01[c]	0.12 ± 0.01[c]
	1:20	0.51 ± 0.02[b]	0.41 ± 0.01[b]
	1:30	0.58 ± 0.01[a]	0.46 ± 0.01[a]
	1:40	0.57 ± 0.01[a]	0.45 ± 0.00[a]
	1:50	0.59 ± 0.01[a]	0.47 ± 0.01[a]
Extraction times	1	0.58 ± 0.01[c]	0.46 ± 0.01[c]
	2	0.69 ± 0.07[b]	0.55 ± 0.06[b]
	3	0.76 ± 0.01[a]	0.61 ± 0.00[a]
	4	0.77 ± 0.04[a]	0.62 ± 0.04[a]
	5	0.77 ± 0.03[a]	0.63 ± 0.02[a]

The values are expressed as means ± standard deviations (n = 3). The values among the levels of each factor in the same column followed by different letters are significantly different at $p = 0.05$ level.

ustiloxins A and B extraction are presented in Table 3. Individually, both ustiloxins A and B content decreased with an increase of methanol concentration from 10 to 100% although, ustiloxins A and B content decreased observably when the temperature was at 100°C, while there were no significant differences in extraction of ustiloxins A and B content with an increase of temperature from 20 to 80°C and extraction time from 10 to 50 min at 60°C, respectively. So both temperature and time were considered to be non-significant factors for the extraction of ustiloxins A and B from rice false smut balls.

Ustiloxins A and B content increased with the increase of extraction pH value from 2 to 6, and achieved maximum at pH 6. Then the content was decreased as the pH value increased from 8 to 10. Both ustiloxins A and B content showed increasing tendency as the material-to-solvent ratio from 1:10 to 1:30 (g/mL), and nearly steady from 1:30 to 1:50 (g/mL). The extraction times as 3 achieved a maximum ustiloxins A and B content.

Among the six factors studied, extraction times and methanol concentration showed high impact on ustiloxin extraction followed by material-to-solvent ratio and extraction pH as compared to other factors (that is, extraction

Table 4. Results obtained under the experimental conditions using an L_{18} OAD.

Run	Content of ustiloxin A (mg/g)	Content of ustiloxin B (mg/g)
1	0.40 ± 0.02^{ef}	0.31 ± 0.00^{ef}
2	0.64 ± 0.01^{b}	0.44 ± 0.01^{b}
3	0.73 ± 0.01^{a}	0.47 ± 0.01^{ab}
4	0.49 ± 0.03^{d}	0.31 ± 0.04^{ef}
5	0.74 ± 0.01^{a}	0.49 ± 0.01^{a}
6	0.45 ± 0.05^{de}	0.35 ± 0.02^{d}
7	0.20 ± 0.02^{g}	0.12 ± 0.04^{j}
8	0.24 ± 0.02^{g}	0.24 ± 0.03^{hi}
9	0.46 ± 0.02^{de}	0.28 ± 0.01^{fg}
10	0.59 ± 0.02^{bc}	0.49 ± 0.04^{a}
11	0.48 ± 0.01^{d}	0.30 ± 0.01^{fg}
12	0.58 ± 0.03^{c}	0.49 ± 0.01^{a}
13	0.62 ± 0.02^{bc}	0.40 ± 0.01^{c}
14	0.57 ± 0.01^{c}	0.46 ± 0.00^{ab}
15	0.46 ± 0.04^{de}	0.34 ± 0.02^{de}
16	0.23 ± 0.04^{g}	0.20 ± 0.01^{i}
17	0.40 ± 0.04^{ef}	0.30 ± 0.02^{fg}
18	0.38 ± 0.01^{f}	0.27 ± 0.01^{gh}

The values are expressed as means ± standard deviations (n = 3). The values in the same column followed by different superscripted letters are significantly different at $p = 0.05$ level.

temperature and time) (Table 3). Therefore methanol concentration (10, 30 and 70%, v/v), extraction pH value (2, 6 and 10), material-to-solvent ratio (1:20, 1:30 and 1:40, g/mL) and extraction times (1, 2 and 3) were selected for further optimization of extraction process of ustiloxins A and B from rice false smut balls by using an orthogonal array design (OAD). The interaction effects of these factors were not found based on our previous central composite design (CCD) experiments (the data were not shown).

Optimization of ustiloxins A and B extraction

As various factors affected the extraction efficiency of ustiloxins A and B, optimization of the operating conditions most is crucial to obtain the higher yield of ustiloxins A and B from rice false smut balls. Based on the single-factor test, methanol concentration, extraction pH, material-to-solution ratio, and extraction times were selected for optimizing the ustiloxin extraction using an OAD of $L_{18}(3^7)$ (Zhang et al., 2009; Guo et al., 2012; Khongsay et al., 2012).

The obtained results (Table 4) show that the maximum content of ustiloxins A and B of the extracts was 0.74 and 0.49 mg/g, respectively. A further orthogonal analysis is given in Table 5. The influence of the extraction factors on ustiloxin A was in order of $A > D > B > C$. In other words, methanol concentration had the dominant effect on ustiloxin A, followed by extraction times, extraction pH and material-to-solvent ratio. The influence of the extraction factors on ustiloxin B was the same as on ustiloxin A (Table 5). ANOVA results show that methanol concentration and extraction times had a significant ($p < 0.05$) effect on the extraction of both ustiloxins A and B (Table 6). Extraction times were not a significant factor for ustiloxin B when the value of p was less than 0.01. The optimum condition obtained for extraction of ustiloxins A and B from rice false smut balls was $A_1B_2C_2D_3$ which means that optimum factors for obtaining the highest content of ustiloxins A and B from rice false smut balls were methanol concentration at 10%, extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3.

Verification of experiments

According to the above analytical results, the optimum conditions for ustiloxin extraction from rice false smut balls were determined as $A_1B_2C_2D_3$, corresponding to methanol concentration at 10%; extraction pH value at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3, respectively. Under the optimum extraction condition, the content of ustiloxins A and B in rice false smut balls was 0.80 ± 0.01 mg/g and 0.57 ± 0.01 mg/g (n = 3), respectively, on a dry weight basis.

In conclusion, this work firstly reported the optimized extraction of ustiloxins A and B from rice false smut balls by using L_{18} orthogonal array design which not only

Table 5. Range analysis of L_{18} orthogonal experiments for ustiloxin extraction.

Compound		Methanol concentration (*A*)	Extraction pH value (*B*)	Material-to-solvent ratio (*C*)	Extraction times (*D*)	Blank 1 (*E*)	Blank 2 (*F*)	Blank 3 (*G*)
Ustiloxin A	K_1	3.42	2.54	2.69	2.48	2.70	2.98	2.81
	K_2	3.32	3.06	3.15	2.63	3.12	2.74	2.87
	K_3	1.92	3.05	2.81	3.53	2.83	2.93	2.97
	k_1	0.57	0.42	0.45	0.41	0.45	0.50	0.47
	k_2	0.55	0.51	0.53	0.44	0.52	0.46	0.48
	k_3	0.32	0.51	0.47	0.59	0.47	0.49	0.50
	R	0.25	0.09	0.08	0.18	0.07	0.04	0.03
	Q	A_1	B_2	C_2	D_3			
Ustiloxin B	K_1	2.50	1.84	1.84	1.81	2.10	2.24	2.14
	K_2	2.37	2.22	2.21	2.03	2.25	2.04	1.97
	K_3	1.40	2.21	2.21	2.43	1.92	1.99	2.16
	k_1	0.42	0.31	0.31	0.30	0.35	0.37	0.36
	k_2	0.39	0.37	0.37	0.34	0.38	0.34	0.33
	k_3	0.23	0.37	0.37	0.41	0.32	0.33	0.36
	R	0.182	0.064	0.061	0.103	0.055	0.042	0.027
	Q	A_1	B_2	C_2	D_3			

K_i^A =Σthe amount of target compounds at A_i; $k_i^A = K_i^A/3$; $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

Table 6. Analysis of variance (ANOVA) of the orthogonal experiments for ustiloxin extraction.

Compound	Source	Sum of squares	d.f.	Mean square	*F*-value	Fa	Significance
Ustiloxin A	*A*	0.235	2	0.117	30.337	$F_{0.05}(2,6)$=5.140	**
	B	0.030	2	0.015	3.907	$F_{0.01}(2,6)$=10.900	
	C	0.019	2	0.010	2.467		
	D	0.108	2	0.054	13.954		**
	Error	0.023	6	0.004			
	Total variation	0.415					
Ustiloxin B	*A*	0.119	2	0.060	19.244	$F_{0.05}(2,6)$=5.140	**
	B	0.016	2	0.008	2.580	$F_{0.01}(2,6)$=10.900	
	C	0.015	2	0.007	2.423		
	D	0.033	2	0.016	5.289		*
	Error	0.019	6	0.003			
	Total variation	0.201					

** Means significance at $p < 0.01$; * means significance at $p < 0.05$.

reduce time but also provide ideal ustiloxin extraction. From the analysis, the optimal design has been determined to be the combination of methanol concentration at 10%, extraction pH at 6, material-to-solvent ratio at 1:30 (g/mL), and extraction times as 3. It will provide the data supporting ustiloxins A and B preparation in large scale to meet their application in agriculture and medicine industry.

Conflict of Interests

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

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Full Length Research Paper

Cytotoxicity of alkaloid fraction from *Sphaeranthus amaranthoides* in A549 cell line

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Sphaeranthus amaranthoides **(sivakaranthai) is a weed in paddy fields found in South Asia. The plant is widely exploited for its pharmacological activity. Previous research showed that the crude extract of this plant possess the antioxidant, anti-inflammatory and antibacterial activity. The current study concentrates on the cytotoxic properties of the alkaloid fraction isolated from the** ***S. amaranthoides.*** **The cytotoxicity was checked using MTT assay method. The assessment of the morphology of the apoptotic cell was done using the fluorescent microscopy. The percentage of apoptosis was investigated dual staining (EB/AO). Nuclear morphology was assessed using propidium iodide. Finally the cell cycle progression was analyzed using flow cytometer. Alkaloids was found to have the highest toxicity towards A549 lung cancer cell lines (IC_{50}= 29.57 µg). The morphology of the lung cancer cells after treatment showed evidence of apoptosis that included blebbing and chromatin condensation. Dual staining showed the evidence of the early apoptosis induced after treatment. The cell cycle analysis indicated that alkaloids were able to induce G2/M phase arrest in lung cancer cells.**

Key words: *Sphaeranthus amaranthoides*, alkaloids, lung cancer cells.

INTRODUCTION

There are about 20,000 species of tropical plants, of which about 1,300 are said to be medicinal and potential sources for screening of anticancer agents (Said, 1999). Some of the plant extracts from these medicinal plants are reported to have potential to be developed as drugs (Manosroi et al., 2006). *Sphaeranthus amaranthoides* Burm. F known as sivakaranthai (asteracea) is distributed in Asia and Africa. The areal parts of this plant are used in the treatment of tumor by the tribal people. This plant possesses anticancer properties; the alkaloid fraction can help to cure the cancer.

Cancer is the disorder caused by the uncontrolled growth of the abnormal cells in the human body. Lung cancer is one of the most commonly diagnosed cancers worldwide making up 12.7% of all cancer cases. It is also the most common cause of cancer death accounting for 18.2% of all cancer related deaths (Ferlay et al., 2008). The literature suggested that natural compounds can be effective in cancer therapy (Reddy et al., 2003; Gullett et al., 2010). One of the main characteristics of the cancer

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is the resistance of cancer cells towards apoptosis which contributes to the ineffectiveness of anticancer therapies (Tang et al., 2010). Apoptosis is characterized by several biochemical and morphological events, such as nuclear fragmentation, internucleosomal DNA fragmentation (Wyllie, 1980), cell shrinkage (Saraste and Pulkki, 2000), chromatin condensation (Lu et al., 2012), formation of apoptotic bodies, loss of plasma membrane asymmetry (van Engeland, 1998), and disruption of mitochondrial membrane (Biasutto et al., 2010). An attempt is made to understand the mechanism(s) followed by alkaloids from *S. amaranthoides*, we have investigated the effect(s) of alkaloids on the A549 cell lines.

METHODOLOGY

Collection of plant material and preparation of crude extract

S. amaranthoides Burm. F (Asteraceae) plant leaves were collected from Tirunelveli district. The Plant was examined and botanically identified by a botanist V. Chelladurai Research Officer-Botany. The collected leaf was shade dried for three weeks to get consistent weight and made in to coarse powder and was used for further studies.

Preparation of ethanolic extract

The leaf powder was soaked in the petroleum ether for two days to dissolve the chlorophyll and then the leaf material was transferred in to the ethanol for five days. On fifth day, leaf material was filtered. The filtered extract was subjected to rotary evaporator to remove the ethanol and to get the concentrated extract in powder form.

Purification of alkaloids from crude extract

The order of solubility for alkaloids: chloroform>acetone>ethanol>methanol>ethyl acetate>ether>n hexane silica gel G60 emulsion was used as a stationary phase in glass column. (1.5 × 50 cm) and chloroform and acetone (10:1, 9:2, 8:3, 7:4) were used as mobile phases. A larger section of the alkaloids are easily soluble in chloroform and relatively less soluble in other organic solvents. The UV-vis spectrophotometer was used to measure the absorbance of the alkaloids on a Cary E-100 Varion type spectrophotometer. The absorbance was measured between 200-800 nm.

HPLC

HPLC system used consisted of a HPLC binary pump, diode array detector (DAD), and an auto-sampler injector compartment (1200 series, Agilent Technologies, Germany). For separation, C-18, 150 mm, 64.6 mm i.d and 5 mm particle size Thermo Hypersil GOLD column was chosen as the reverse phase while the mobile phase was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with the gradient setting of solvent B: 5% (5 min), 5-90% (60 min), 5% (4 min) at a flow rate of 1 ml/min. Detection wavelengths were both set at 280 and 360 nm with constant injection volume of 20 ml. A 3200 QTrap LC/MS/MS system was used for the mass spectrometry analysis, with the iron source and voltage maintained at 500uC and 24.5 kV for negative ionization, respectively. The nitrogen generator was set at 60 psi curtain gas flow, 60 psi exhaust gas flow, and 90 psi source gas flow. The scanning modes selected were enhance mass spectrometer (EMS) and enhance ion product (EIP) for full scan mass spectra that ranged from mass to charge ratio (m/z) of 100-1200.

Cell culture conditions

The A549 lung adenocarcinoma cell line was procured from National Centre for Cell Science (NCCS), Pune, India with the passage number of 11. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, along with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Cells were grown in 75 cm^2 cultures and after a few passages, cells were seeded in a 96-well plate. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using trypsin-EDTA solution.

Cell proliferation assay

Proliferation of A549 cells was assessed by MTT assay (Safadi et al., 2003). Cells were seeded into 96-well plates at 5×10^4 cells per well 24 h before treatment. After treatment with drugs, cell proliferation was determined using MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 15 µl (5 mg/ml) MTT solutions was added to each well, and incubated at 37°C for 4 h, after which the MTT solution was discarded and 200 µl of dimethylsulfoxide (DMSO) was added to dissolve the crystals of formazan dye by pipetting up and down. Spectroscopic absorbance of each well was measured at 570 nm using an ELISA reader (BIORD) at 570 and 630 nm as background using a microplate reader (Synergy H1 Hybrid). The IC_{50} value was determined from the dose response curve.

Ethidium bromide/acridine orange (dual staining)

Ethidium bromide/acridine orange staining was carried out by the method of Gohel et al. (1999). A549 cells were plated at a density of 1×10^4 in 48-well plate. They were allowed to grow at 37°C in a humidified CO_2 incubator until they were 70-80% confluent. Then cells were treated with 15.625 and 31.25 µg/ml (selected based on the IC_{50} concentration) of alkaloid fraction for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and drug treated were mixed with 100 µl of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells were counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

Assessment of nuclear morphology after propidium iodide staining

Propidium iodide staining was carried out by the method of Chandramohan et al. (2007). A549 cells were plated at a density of 1×10^4 in 48-well plates. They were allowed to grow at 37°C in a humidified CO_2 incubator until they are 70-80% confluent. Then cells were treated with 15.625 and 31.25 µg/ml of alkaloid for 24 h.

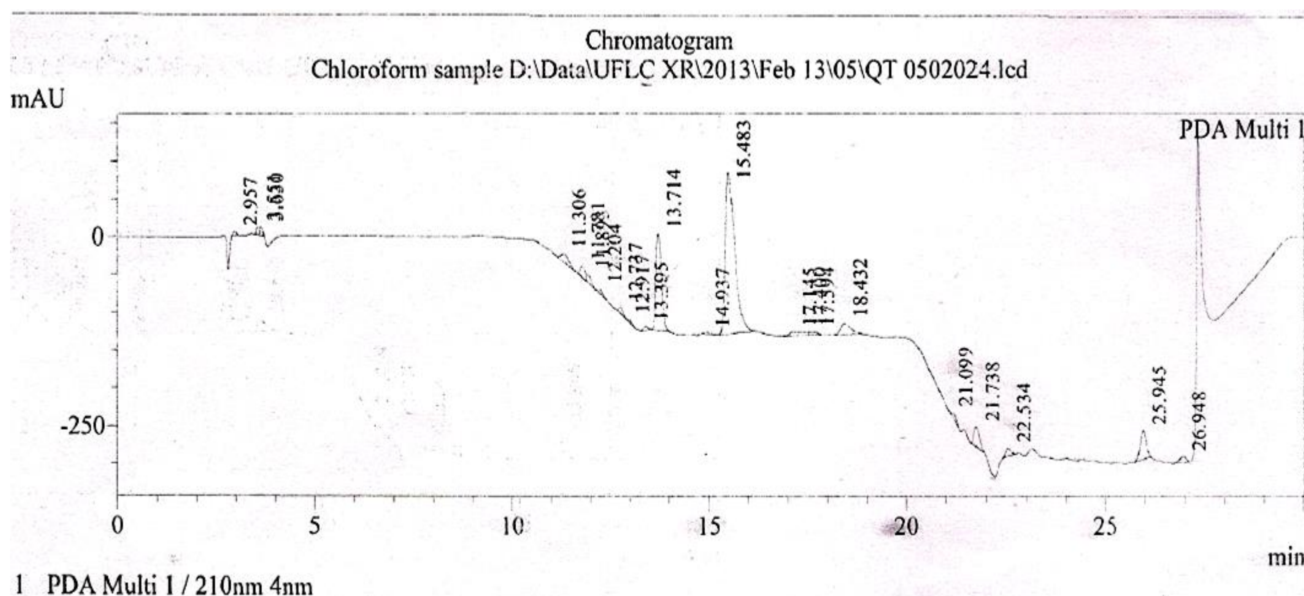

Figure 1. Graph showing the alkaloids retention time (HPLC chromatogram).

Culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature, before fixing in methanol : acetic acid (3:1 v/v) for 10 min, and stained with 50 µg/ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1×10^3 cells were counted for assessing apoptotic cell death.

Cell cycle analysis

To investigate the effect of alkaloid fraction on the cell cycle distribution, A549 cells (1×10^5 cells/ml) were treated with 15.625 and 31.25 µg/ml cultured for 24 h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 µg/ml propidium iodide (PI) and 0.1 mg/ml RNase A followed by shaking at 37°C for 30 min. The stained cells were analyzed with flow cytometer at Ramachandra medical collegeat Chennaiand the data were consequently calculated using WinMDI 2.9 software (Tu et al., 2004).

Statistical analysis

Data was expressed as mean ± S.E.M and analyzed by Tukey's test to determine the significance of differences between groups. A p value lower than 0.05, 0.01 or/and 0.001 was considered to be significant.

RESULTS

The HPLC analysis of the sample revealed 20 different peaks with different retention times in that two major peaks were observed (Figure 1, Table 1).

Cell cytotoxicity assay using MTT method

The A549 lung adenocarcinoma cells were treated with various concentrations (5 - 640 µg) of alkaloid fraction and subjected to MTT assay. As shown in Figure 2, treatment of A549 cells with the drug resulted in significant dose - dependent reduction in cell growth ranging from 12.29±0.01 to 87.98±0.003 after 24 h. The IC_{50} value was found to be 29.57 µg (Table 2), the results obtained reveal that the alkaloids from the plant have strong anticancer activity towards A549 cells. Figure 3 shows the morphological changes due to the alkaloids treatment with various concentrations. The cell morphology in control was observed normal and number was more but in the alkaloid treated cell lines in 20 µg/ml showed spherical shaped. At 80 µg/ml, the number was reduced and the morphology was spherical shaped. Finally with 320 µg/ml, the cells were dead.

Quantification of apoptosis using acridine orange and ethidium bromide assay (apoptosis)

Acridine orange/ethidium bromide (AO/EB) was done to evaluate the type of cell death induced by alkaloid fraction in A549 cells; the morphological variations after double staining were investigated. Live cells stained with AO emitted green fluorescence. Early apoptotic cells had fragmented DNA which exhibited intense green colored nuclei. Late apoptotic and necrotic cell's DNA were fragmented and stained orange and red. From the data it was clear that with increase in the concentration of drug,

Table 1. HPLC DATA (peaktable).

Chl 210 nm 4 nm			
Peak#	**Ret. time**	**Area**	**Area %**
1	2.96	28141	0.41
2	3.55	55842	0.81
3	3.63	46166	0.67
4	11.31	144820	2.11
5	11.78	128760	1.87
6	11.89	79199	1.15
7	12.20	39944	0.58
8	12.74	51948	0.76
9	12.92	14380	0.21
10	13.39	43506	0.63
11	1371	1245617	18.11
12	14.94	54107	0.79
13	15.48	3632779	52.82
14	17.15	62919	0.91
15	17.40	75742	1.10
16	17.59	59726	0.87
17	18.43	259250	3.77
18	21.10	50726	0.74
19	21.74	218746	3.18
20	22.53	96601	1.40
21	25.95	402276	5.85
22	26.95	86320	1.26
Total		6877515	100.00

Shaded values indicate 2 two major peaks which were highest

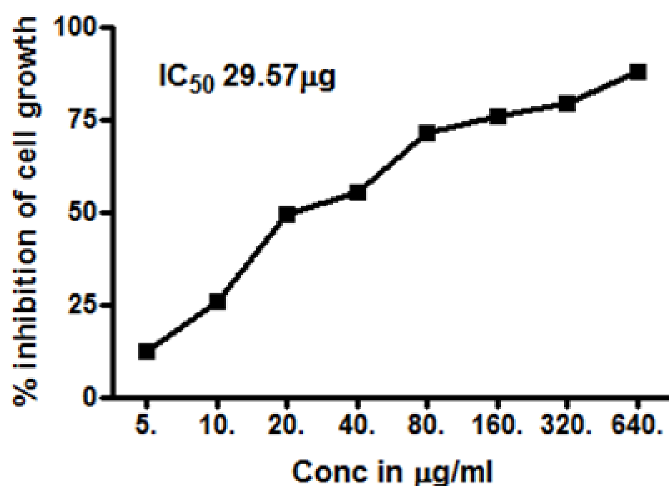

Figure 2. Inhibitory effect of the alkaloid fraction treatment on A549 lung adenocarcinoma cells (MTT ASSAY).

Table 2. Cytotoxicity of alkaloid fraction.

Concentration (µg/ml)	% inhibition of cell growth
5	12.29 ± 0.011
10	26.02 ± 0.002
20	49.28 ± 0.005
40	55.39 ± 0.004
80	71.55 ± 0.005
160	75.76 ± 0.003
320	79.30 ± 0.002
640	87.98 ± 0.003

the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with 15.625 and 31.25 µg/ml of drug was drastically increased ($p< 0.001$) to 44 and 70%, respectively. The study reveals that the alkaloids triggered morphological changes in treated A549 cells that indicated possible induction of apoptosis upon treatment in a time dependent manner (Figure 4). The presence of intercalated AO within fragmented DNA indicates early apoptosis. 24 h after the treatment with alkaloids, blebbing and nuclear chromatin condensation were noticeable. Late apoptosis was indicated

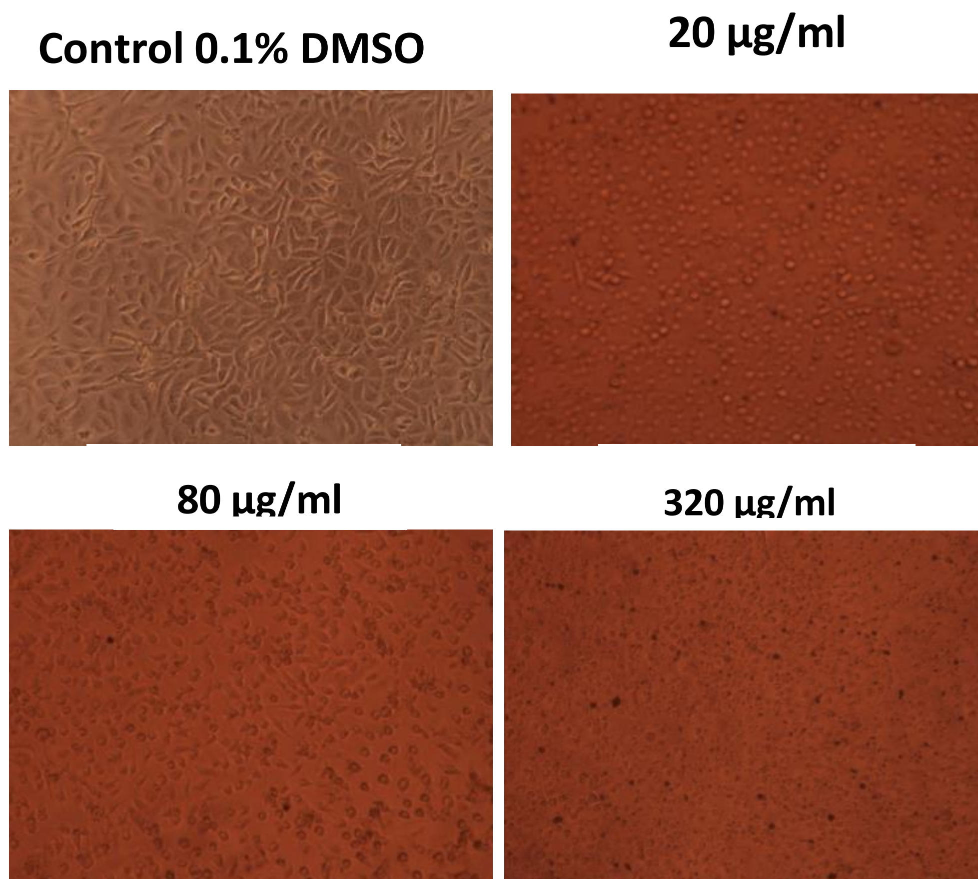

Figure 3. Morphological changes induced by different concentrations of alkaloid fraction.

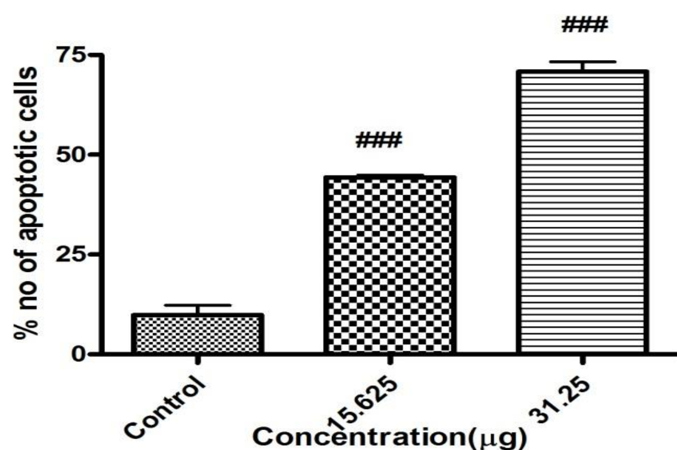

Figure 4. Bar graph showing the percentage number of apoptotic cells.

indicated by the presence of orangish red colour due to the binging of the AO to denatured DNA as observed after 48 h of treatment with alkaloids (Figure 5).

Propidium iodide - nuclear fragmentation assay

Apoptosis was further confirmed by analyzing the nuclear morphology of drug-treated A549 cells. Nuclear morpho-logy was evaluated with membrane-permeable PI stain. The treated cells contained more apoptotic cells while comparing to untreated cells. There was charac-teristic nuclear fragmentation of nuclei in treated A549 cells whereas the untreated control cells did not show any nuclear fragmentation (Figure 6). The apoptotic cells displayed characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. The percentage of apoptotic nuclei after treatment with 15.625 and 31.25 µg/ml of drug increased enormously ($p<0.001$) to 53 and 69%, respectively (Figure 7).

FACS analysis

Flow cytometric analysis of the cell cycle was performed to determine the ability of alkaloids to induce cell cycle arrest and apoptosis in A549 cells. After 24 h of incubation,

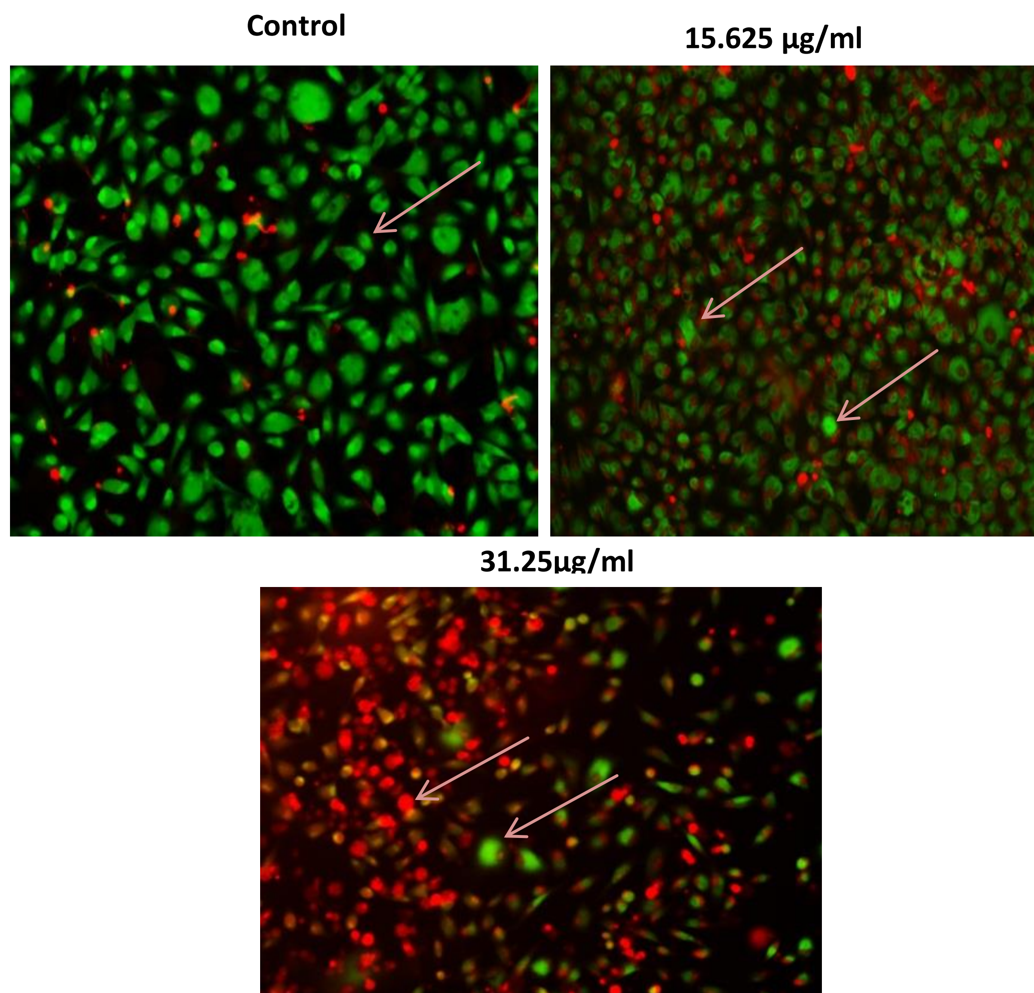

Figure 5. Induction of apoptosis by the drug in A549 cells.

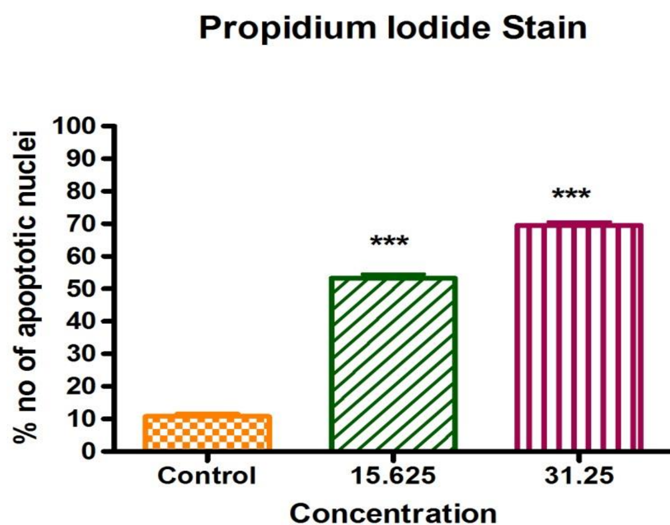

Figure 6. Percentage of apoptotic nuclei.

stability in all the cell cycle populations is generally noticed and compared with the control cell line without treatment. Alkaloid fraction treatment increased the cells in the G2/M phase from 10.45 to 28.32% in 15.625 µg treated cells and 48.79% in 31.25 µg treated cells, respectively. Similarly accumulation of cells in the sub G1 phase from 4.56 to 9.14 and 21.32% in the two treated concentrations was also observed (Figure 8). An increased cell population in the G2/M and sub G1 phase with a concomitant decrease in the G0/G1 and S phase compared to the untreated cells suggest that the alkaloid fraction inhibited the cell cycle progression in G2/M phase and subjected the cells to apoptosis which is evident from cell accumulation in sub G1 phase.

DISCUSSION

In the present study, we report the type of apoptosis induced

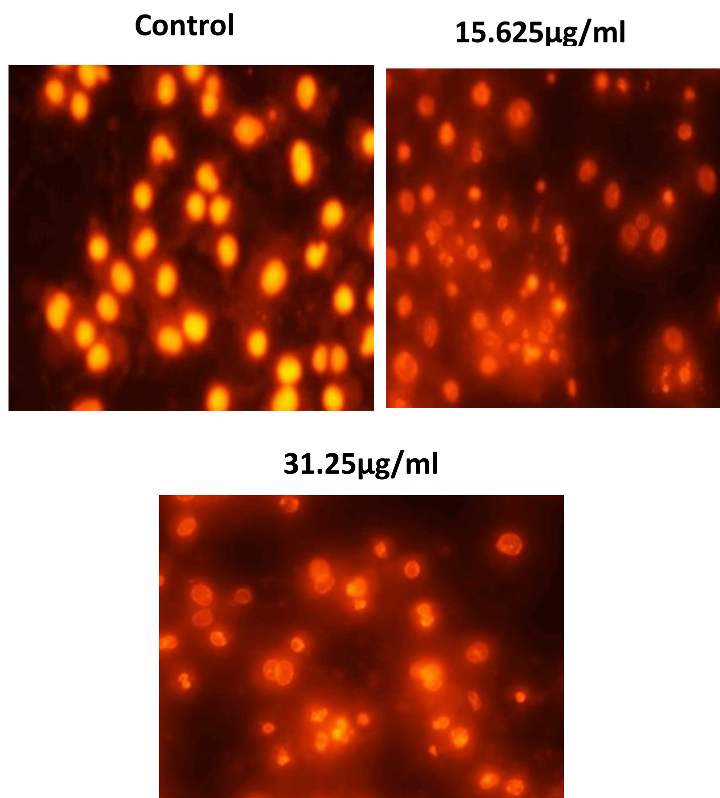

Figure 7. Nuclear localization of A549 cells by propidium iodide staining.

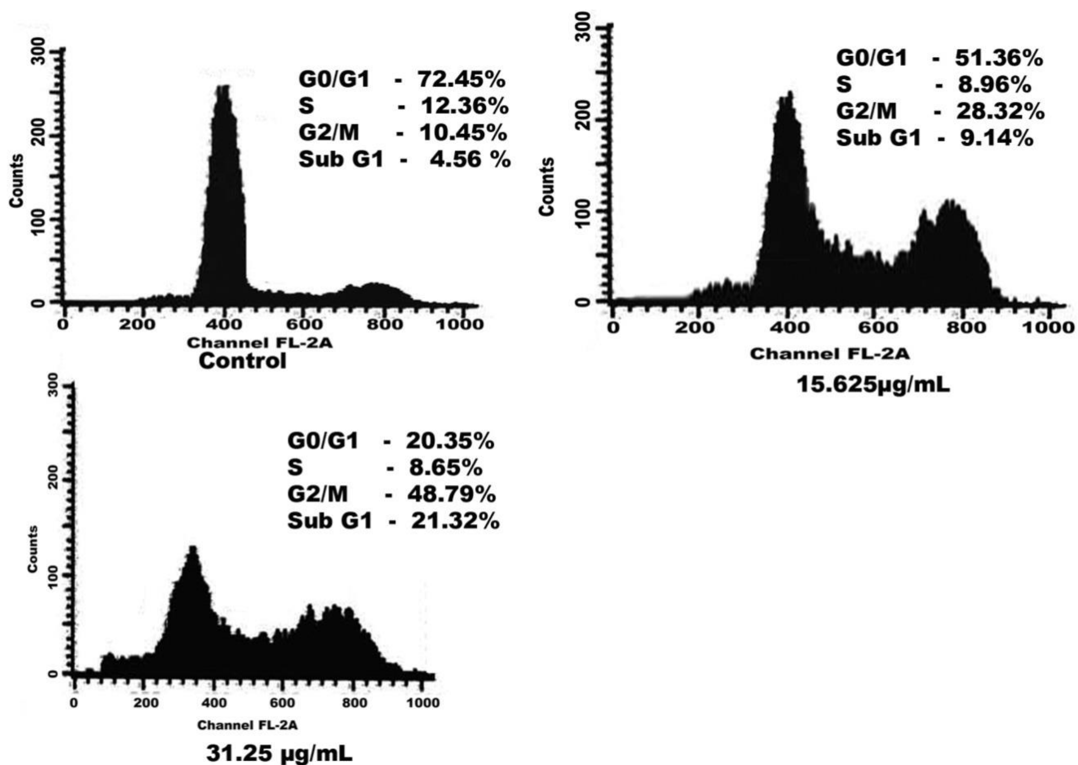

Figure 8. FACS analysis.

by alkaloids from *S. amaranthoides* in A549 cell lines. *S. amaranthoides* is well known plant for the different pharmacological activities which includes hepatoprotective, wound healing, antibacterial activity, anti-inflammatory and antioxidant activity. Cytotoxic activity of *S. amaranthoides* was not reported earlier. In the current report, the type of apoptosis and molecular changes occurred were reported. Depending on the current study the alkaloid fraction showed a good cytotoxic effect on A549 cell line with ic_{50}- 29.78 µg/ml. The alakloids are considered to be active against cancer cell *in vitro* when the IC_{50} is within the concentration range of 1-50 µM (Boik, 2001). Dose and time dependent (Figure 2) cytotoxicity of alkaloids on cancer cell lines were observed. This suggested that treatment with alkaloids inhibited the growth and reduced the viability of these cells. The induction of apoptosis has been described as a standard and best strategy in anticancer therapy (Russo et al., 2006; Hannun, 1997).

The phase-contrast microscopical studies help to identify the early stage of apoptosis which are characterized by the reduced size of the cells, blistering and blebbing of the nuclear membrane (Xu et al., 2004; Willingham, 1999). As seen in the dose and time-dependent treatment (Figure 8), cells get started to separate from the surface of the culture plates. Condensation of the nuclear chromatin is one of the apoptotic character that leads to breakup of the chromatin leading to nuclear fragmentation (Willingham, 1999).

In the present study the detection of early and late apoptosis was performed with dual staining. Based on the results, we have found that alkaloids induced apoptosis with a significant increase the concentration 29.57 µg/ml in A549 cell lines. This was proven with the signs of nuclear fragmentation and chromatin condensation. This can be further supported by the results of cell cycle distribution which showed the accumulation of cells in the S phase and the decrease of cell percentage in the G0/G1phase.

Accumulation of cells in S phase may have contributed to the high level of apoptosis in A549 cells (Pozo-Guisado et al., 2002). The cells blocked in the S phase are now a check point that inhibits the replication due the DNA damage which caused a decrease in cell survival (Bunch and Eastman, 1997; Shi et al., 2001). Hence the above data suggests that the alkaloids altered the cell cycle in a dose-dependent manner, and this could explain the observed correlation cell growth inhibition, cell cycle blockade and cell death (Arora et al., 2011).

Conclusion

The current study states that alkaloids possess strong inhibitory effects on cell growth and is capable of inducing apoptosis in A549 cells. Alkaloids also appear to affect the cell cycle which can induce apoptosis. The present findings provide valuable information in the development of natural compounds for use in cancer therapy.

Conflict of Interests

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

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Full Length Research Paper

Simple and enhanced production of lignocellulosic ethanol by diluted acid hydrolysis process of pineapple peel (*Ananas comosus*) waste

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Ethanol can be produced from a fermentation process using raw materials obtained from highly economically important plants such as corn, cassava and sugarcane, and used as an alternative energy source. These economical plants are being used less because their initial cost is still increasing. However, lignocellulosic ethanol production can alternatively be done from agricultural wastes such as corn stover, sugarcane bagasse and rice straw. In this work, a solution of hydrolyzed pineapple peel was the substrate and was converted to ethanol via batch fermentation. The preparation and characterization of the cellulose obtained from the modified TAPPI T203 test method for the enhancement of the ethanol production was investigated. The results show that the FTIR spectra of their removable lignin and hemicellulose disappeared at 1590, 1475, 1250 and 1164 cm^{-1}, respectively. The percentage of cellulose obtained was 20.44. The maximum percentage yield of total reducing sugars in the diluted acidic hydrolysis of pineapple peel by 0.2 M H_2SO_4 was 82.10±2.30. The fermented broth using *Saccharomyces cerevisiae* TISTR 5048 gave the highest percentage of bioethanol yield which was 65.27±2.45%. This process is not complicated, simple and low cost for ethanol production industries.

Key words: Lignocellulosic ethanol, diluted acid hydrolysis, pineapple peel waste, total reducing sugars.

INTRODUCTION

Currently, a continuous increase in oil price as a result of the global energy crisis is an urgent problem awaiting a solution in many countries. Renewable energy is being discussed, including alternative raw materials, such as municipal waste and waste paper or particular crops. The economical use of energy is the first issue which can

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decrease the energy expenditure by reducing crude petroleum fuel imported from the Organization of Petroleum Exporting Countries (OPEC). In addition, alternative fuels from renewable resources are subjects for energy conservation which can replace petroleum fuel resources (Wyman, 1994). A renewable energy source such as ethanol was used in Brazil and the USA by mixing fuel oils to increase the octane number of fuel oil (Laluce, 1991). Ethanol can be produced through fermentation of lignocellulosic biomass such as sugar cane and corn using microorganisms (Morais et al., 1996).

Lignocellulosic biomass is a potential source of cheap sugars for producing fuels and chemicals, and a pretreatment stage is essential to make the cellulose accessible to hydrolysis by a dilute acid (Mohagheghi et al., 2004). Cazetta et al. (2007) studied the utilization of lignocellulosic biomass which has been closely associated with a new technological concept, the so called Biorefinery. Therefore, ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the utilization of fossil fuels (Dale, 1999).

Thailand is an agricultural country which has plentiful agricultural wastes. They can be used as material sources for ethanol production such as sugarcane bagasse, cassava stem, corn stover and rice straw. Therefore, Lin and Tanaka (2006) have developed a new material which has a high content of lignocellulosic biomass and possesses a reduced demand for supplying the ethanol production process, the so called lignocellulosic ethanol. Pineapple peel is one of the agricultural wastes which has lignocellulosic biomass. Although most of lignocellulosic biomass is composed of 38 to 50% cellulose, 23 to 32% hemicellulose, and 13 to 30% lignin (Sierra et al., 2008), pineapple peel is an interesting biomass resource for lignocellulosic ethanol production because there is a lot of peel waste. However, the production of lignocellulosic ethanol is a relatively complicated process. Reddy and Reddy (2005) have developed the transformation of biological resources as rich energy crops requiring the optimum conditions for conversion as lignocellulosic ethanol by fermenting organisms. Additionally, aqueous solutions of ethanol should be concentrated for obtaining hydrous ethanol (Maiorella et al., 1984). The ethanol in gasoline was used as an oxygenated fuel. Cardona and Oscar (2007) studied the complexity of this process partly to explain why fuel ethanol has not played a leading role in comparison to cheaper oil derived fuels. Due to rising environmental concerns and the periodic crises in some of the larger oil exporting countries, it has become a viable and realistic alternative in the energy market (Bayrock and Ingledew, 2001).

In addition, the main components of lignocellulosic biomass and type of microorganisms can also affect lignocellulosic ethanol production (Boerjan et al., 2003). Thomas and Rose (1979) have developed non-pretreated lignocellulosic biomass and *Saccharomyces cerevisiae* for use in lignocellulosic ethanol production with the advantage of simple ethanol production. It can grow in aerobic conditions and is used in the baking and brewing industries (Lynd et al., 1991; Narendranath and Power, 2005). Grosz and Stephanpoulos (1990) have developed ethanol producing organisms used in industrial processes. In previous works, lignocellulosic ethanol from pineapple peel by the enzymatic hydrolysis process via Simultaneous Saccharification and Fermentation (SSF) was studied (Itelima et al., 2013). Although the enzymatic hydrolysis process can give high yield bioethanol, it is difficult to perform it at a large scale for ethanol production industries as a result of the complicated procedure and high cost. The aim of this study was to produce the lignocellulosic ethanol via batch fermentation from the dilute acid hydrolysis process of pineapple peel waste. This work was conducted to provide an added value to this waste for the canned fruit industries. The main components (lignin, hemicellulose and cellulose) were characterized by Fourier Transform Infrared (FTIR) and TG/DTA techniques. In addition, the contents of total reducing sugars and lignocellulosic ethanol obtained were investigated by the spectrophotometric technique and gas chromatography - flame ionization detector (GC-FID), respectively.

MATERIALS AND METHODS

Fresh pineapple peel was collected from the Food Service Center in Khon Kaen University (KKU), Khon Kaen province, Thailand. Firstly, the pineapple peel was washed thoroughly with distilled water, minced and dried at 60°C in a hot-air oven. After that, it was ground and sieved to obtain particle sizes of less than 500 µm. This sample was stored in a plastic box before use and then characterized by a FTIR spectrometer (Spectrum One; Perkin Elmer, Germany) with the KBr pellet method.

Determination of main components

The main components in the pineapple peel such as lignin, hemicellulose and cellulose were determined by a Perkin Elmer Thermogravimetry (TG) (Pyris Diamond TG/DTA 6300, Germany) with temperature ranging from 30 to 830°C and heating rate of 10°C/min under nitrogen atmosphere (Nishiyama et al., 2002).

Removal of main components

This method was modified from the Technical Association of Pulp and Paper Industrial T203 test method (TAPPI, 1994-1995). Briefly, 10 g of raw pineapple peel powder was extracted with the solvent, a mixture of hexane: methanol of 2:1 by volume to remove ester compounds by shaking at 180 rpm for 30 min and drying in a fume hood for solvent disposal (assigned as Sample I). The lignin removal was done by soaking Sample I in 150 mL deionized water with the addition of 1.5 g $NaClO_4$ and 10 drops of 18 M CH_3COOH

in a water bath at 70°C for 1 h. Then, it was washed thoroughly with distilled water, dried in an oven at 80°C for 1 h and weighed (assigned as Sample II). Then, the powder of Sample II had the hemicellulose removed with soaking in 0.25 M NaOH for 24 h and then it was boiled at 70°C for 1 h as modified from the TAPPI T203 test method. It was then washed thoroughly with distilled water, dried in an oven at 60°C and weighed to achieve dried cellulose (assigned as Sample III) at the end. All three samples were characterized by using a FTIR spectrometer with the KBr pellet method.

Diluted acid hydrolysis

Ten (10) g of samples were weight hydrolyzed with 100 mL 0.2 M H_2SO_4 using an electrical autoclave (All American Pressure Sterilizer, U.S.A.) at 120°C, 15 psi for 90 min. The working conditions for diluted acid hydrolysis were studied (Xu et al., 2003). Then, the hydrolyzed solution obtained was filtered through filter paper. The total reducing sugars of the hydrolyzed solution were determined according to the dinitrosalicylic acid method at wavelength 570 nm by a UV-VIS spectrophotometer (Agilent 8453 UV-Visible Spectroscopy System, Germany) (Miller, 1959).

Fermentation

A pure yeast strain of *S. cerevisiae* TISTR 5048 in this experiment was purchased from the Microbiological Resources Center, Thailand Institute of Science and Technological Research (TISTR), Pathum Thani Province, Thailand. For the batch fermentation process, the hydrolyzed solution of pineapple peel was neutralized to pH 7.0 using 2.0 M NaOH and filtered through filter paper. Then, this solution was added into the synthetic medium (consisting of 1.0 g/L yeast extract, 1.0 g/L $MgSO_4$, 2.0 g/L $(NH_4)_2SO_4$ and 0.5 g/L KH_2PO_4 in 1 L of distilled water) (Brown et al., 1981). After that, it was sterilized using an electrical autoclave at 120°C, 15 psi for 30 min. Then, 10.0 mL of *S. cerevisiae* TISTR 5048 broth was loaded into this medium. Finally, the batch fermentation was carried out by a rotary shaker with speed 150 rpm at 30°C for 72 h. by sampling every 6 h.

Ethanol analysis

The fermented broth was obtained, centrifuged at 3000 rpm for 10 min and filtered through a 0.45 µm filter membrane. The bioethanol was monitored by a (GC-FID) (TraceGC, Thermo Finnigan, Italy) using a DB-5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The temperature of the injector was set at 250°C. The flame ionization detector was kept at 280°C. The temperature was programmed at 50°C for 2 min, from 50 to 100°C at 10°C/min, then held for 2 min at 100°C. The internal standard used was n-butanol (Caylak and Vardar, 1998).

RESULTS AND DISCUSSION

Identification of main components in pineapple peel

The IR spectra of the raw pineapple peel from Figure 1a for mode assignment showed O-H stretching of acid and methanol in reducing sugars at 3500 to 3200 cm^{-1}, $C–H_n$ stretching of alkyl, aliphatic and aromatic occurring at 1750 cm^{-1}, C=O stretching of ketone and carbonyl occurring at 1590 cm^{-1}, C=C stretching of aromatic skeletal mode in lignin occurring at 1475 cm^{-1}, C-O-C stretching of aryl-alkyl ether linkage occurring at 1250 cm^{-1} and C-O-C stretching vibration in hemicellulose occurring at 1164 cm^{-1} (Yang et al., 2007). These confirmed main components were present in the raw pineapple peel such as the reducing sugars in cellulose and hemicellulose, the ester compounds, and the lignin portion. From the main components removal with the modified TAPPI T203 test method, Sample I had alkyl, aliphatic and aromatic compounds removed from raw pineapple peel as shown in Figure 1b in which there is no peak at 1750 cm^{-1}. In Figure 1c of Sample II spectra peaks disappeared at 1590 and 1475 cm^{-1} due to the absence of lignin. In Sample III due to the hemicellulose removal the peak of arabinose disappeared at 1250 and 1164 cm^{-1} (Figure 1d). The characterization of the main components was successfully achieved by FTIR at each step.

Determination of main components by the TG/DTA technique

The DTG curve in Figure 2a shows that the first event was moisture removal (1) up to around 100°C followed by the second, third and fourth events around 150 to 300°C were the evolution of hemicelluloses (2) and cellulose (3) degradation, respectively. Degradation of lignin took place slowly over a wide temperature range and rose to a higher temperature (4) (Yang et al., 2006; Yang et al., 2007). The TG curve shows a maximum percentage of weight loss occurring in the temperature range of 150 to 450°C. The first step that could be attributed to decomposition begins with moisture about 100°C. The second mass loss step was hemicellulose degradation of 9.50±0.77%. It has an amorphous structure and linear polymer structure with short side chains which are easier to remove than cellulose and some hydrocarbons at the lower temperature. The third step, related to cellulose, mainly consists of a semicrystalline arrangement of chains associated with others which is a strong structure degrading by 21.16±0.73% and the final step was 42.11±0.85% for the lignin degradation because it is complex and has a strong structure of a phenolic polymer covering the polysaccharides of the cell walls as shown in Figure 2b (Wanitwattanarumlug et al., 2012).

Determination of main components by removal processes

The modified TAPPI T203 test method can be used to determine the main components in pineapple peel. It can be performed from the weight loss of three samples by

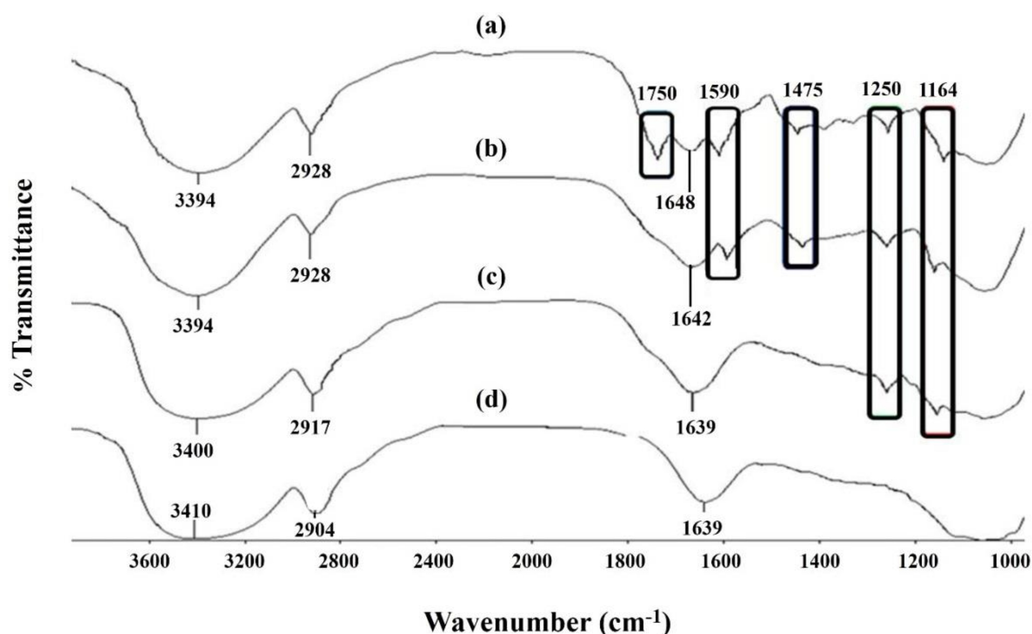

Figure 1. The IR spectra of (a) raw pineapple peel, (b) Sample I = lignin + hemicellulose + cellulose, (c) Sample II = hemicellulose + cellulose and (d) Sample III = cellulose. The mode assignment of the functional groups in each main component was removed.

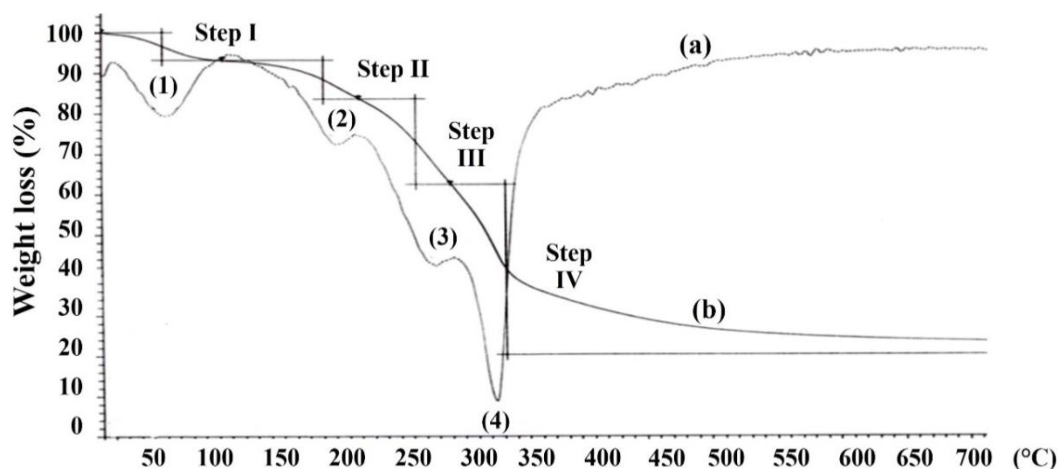

Figure 2. The thermal gravimetric analyzer (TG/DTA) curve (a) and TG curve (b) show typical weight losses of components in raw pineapple peel monitored in the range of 30-830 °C. Step I: moisture removal (I), Step II: hemicellulose degradation (II), Step III: cellulose degradation (III) and Step IV: lignin degradation (4).

the classical gravimetric method. It is interesting to note that raw pineapple peel was composed of 9.43±1.51% hemicellulose, 20.44±1.45% cellulose and 41.21±3.07% lignin. Figure 3 shows a comparison of the main components between removal processes and the TG/DTA technique showing that there is no significant difference between the removal processes and the TG/DTA data.

Determination of total reducing sugars

The standard curve for glucose solution was achieved between 0.01 to 0.10%, w/v by using the dinitrosalicylic acid method by a UV-VIS spectrophotometer. The percentage of total reducing sugars in all of the hydrolyzed samples is shown in Figure 4. The hydrolyzed solution of Sample II and Sample III gave the total

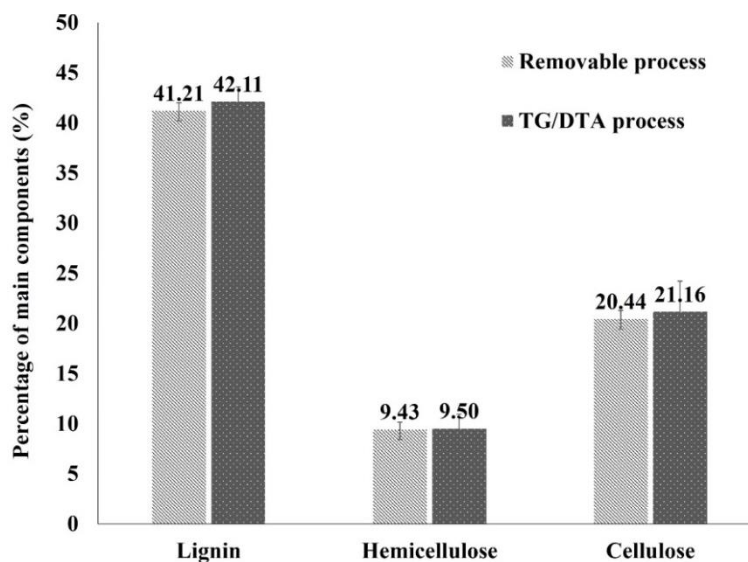

Figure 3. The percentage of components in raw pineapple peel obtained from each removal process compared with TG/DTA data.

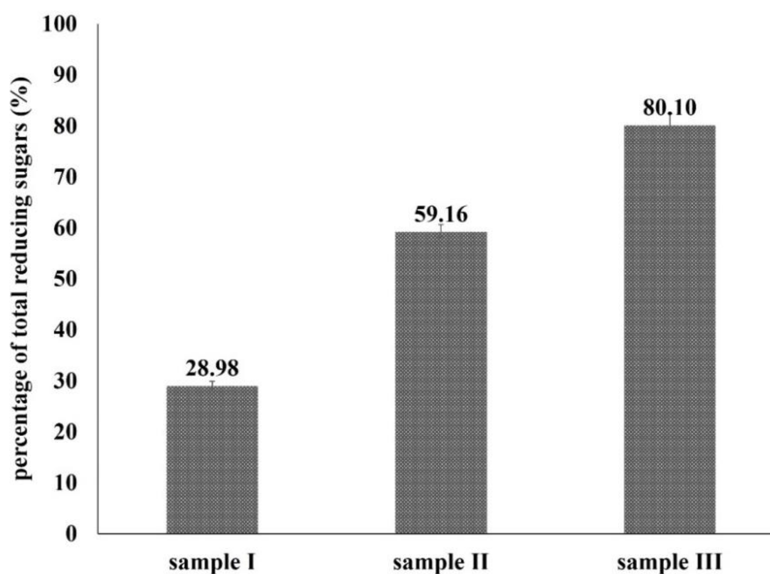

Figure 4. The percentage of the total reducing sugars obtained from the hydrolyzed pineapple peel samples. Sample I = lignin + hemicellulose + cellulose; Sample II = hemicellulose + cellulose; Sample III = cellulose.

reducing sugars of 59.16±1.48 and 82.10±2.30%, respectively. For the hydrolyzed solution, Sample III had the highest amount of total reducing sugars which was converted from all glucose, because of the highest amount of cellulose from the removal process. Normally, the structure of the cellulose is linear chain polymers which can be hydrolyzed with diluted sulfuric acid more easily than the others (Updegraff, 1969). The hydrolyzed solution of Sample II also contained some glucose and other reducing sugars from the hydrolyzed hemicellulose. In fact, the structure of hemicellulose is long and has many branches which could be a blocker to diluted sulfuric acid hydrolysis (Ebringerova et al., 2005). While the hydrolyzed solution of Sample I gave 28.98±0.96%

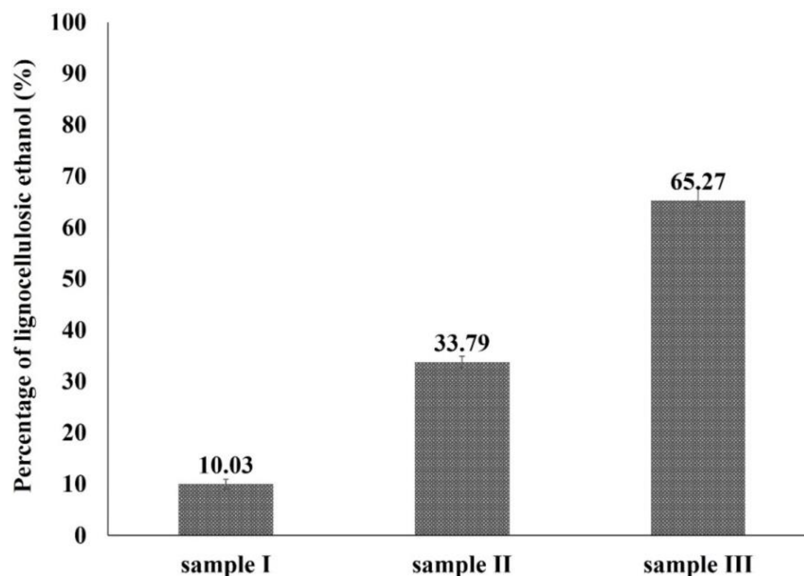

Figure 5. The percentage of bioethanol obtained from three pineapple peel samples via batch fermentation.

of total reducing sugars; it was quite low because the effect of the amount and the complicated structure of lignin, including long and many branches of hemicellulose.

Determination of lignocellulosic ethanol

The standard curve of ethanol was obtained in the range of 0.01 to 0.09% v/v and the percentage of lignocellulosic ethanol obtained from each fermented broth sample is shown in Figure 5. The fermented broth of Sample III gave the highest bioethanol of 65.27±2.45%. This sample contained most glucose for producing bioethanol fermented by *S. cerevisiae TISTR 5048*, which was better than others and there was no interference from other components (Lui and Shen, 2008). The percentage of lignocellulosic ethanol in the fermented Sample II was 33.79±1.16%. The other reducing sugars (such as xylose and arabinose) from this sample could not be converted to ethanol by *S. cerevisiae* TISTR 5048 (Marek et al., 2007). Finally, the fermented broth of Sample I attained 10.03±0.93% only. The lowest percentage of lignocellulosic ethanol obtained was due to inhibition from the high content of the lignin. A regular elevation in production of lignocellulosic ethanol was observed until 18 h of batch fermentation and declined thereafter. *S. cerevisiae* TISTR 5048 has been promising in utilization of maximum fermentable glucose presented in hydrolyzed lignocellulose which is reflected in higher bioethanol production and a greater yield. The lignocellulosic ethanol production from pineapple peel can be enhanced with the preparation of cellulose in the removal processes using modification from the TAPPI T203 test method. However, our process may give less ethanol yield than others processes. Nonetheless, it was found that this diluted acid hydrolysis process for this pineapple peel waste is quite simple, uncomplicated and low cost. So it can be applicable in the ethanol production industries.

Conflict of Interests

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

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Full Length Research Paper

Toxicological aspects of geophagia in pregnancy: Putative molecular mechanism involving oxidative stress

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A number of studies have demonstrated that geophagia can be beneficial and harmful. However, toxicological aspects of this earth, soil, clay eating habit are not fully investigated. In addition, low levels of antioxidants have been observed in pre-eclamptic women. Still, the molecular mechanism implicated oxidative stress in geophagia inducing pre-eclampsia has not been elucidated. Despite its absorbent properties in detoxifying phytotoxin, lowering the incidence of nausea, natural clay still remains toxic for human consumption in normal and long term conditions. Our previous studies have shown the implication of oxidative stress in pre-eclampsia characterized by maternal hypertension. Biomarkers of oxidative stress such as lipid peroxides (LPO), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and 8-OHdG correlated with the extent of oxidative stress in pre-eclampsia. Fenton reaction induced by the presence of iron produces a more powerful reactive oxygen species (OH radical) which may contribute to the exacerbation of this status; while, clay eating is believed to protect pregnant women from pre-eclampsia by inhibiting iron uptake from gastrointestinal environment and so reducing the risk of production of OH radical during pregnancy where iron deficiency has been observed, it may be harmful however in iron overload conditions. We have demonstrated that cellular hepato-carcinoma can be indeed induced in rats being iron-loaded by ferrocene supplementation of their diet. Taking into account this possibility, we are postulating that during pre-eclamptic pregnancy, a number of factors including oxidative stress, activated macrophages and activation of xanthine oxidase (XO) can exacerbate this pregnancy complication. In addition, the availability of free iron from soil may as catalytic trace element involve in Harber-Weiss and Fenton reactions producing more ROS/RNS and especially OH radical. We are therefore suggesting that the subsequent activation of the nuclear factor NF-kB by ROS/RNS generated from activated macrophages stimulates the induction of COX-2. This enzymatic induction leading to the expression of inflammatory cytokines could be one of the molecular mechanisms underlying pre-eclamptic conditions. Supplementation of zinc or eating clay containing zinc could overcome such pregnancy complication.

Key words: Geophagia, pregnancy, molecular mechanism, oxidative stress, Haber-Weiss and Fenton reactions, superoxide dismutase (SOD), glutathione peroxidase (GPX), OH radical, 8-hydroxyl deoxyguanosine (8-OHdG).

INTRODUCTION

According to Coreil et al. (2000), geophagia may be stimulated by dietary deficiencies and appears to be ubiquitous. An overview of potential benefits and negative consequences of geophagia has been outlined by Young (2008) who indicated the mechanism of useful micronutrients from soil such as iron, zinc or calcium and also that of poisonous elements; such as lead, mercury, etc. A body of information has been provided by a large number of studies (Lacey, 1990; Abrahams and Parsons, 1996; Njiru et al., 2011).

Iron and zinc for example obtained through geophagia or normal diet can lose their beneficial effect if they are absorbed by clay. Their uptake from gastro-intestinal tract can indeed cause iron and zinc deficiencies with subsequent consequences respectively on the erythropoiesis inducing a syndrome hypochromic anemia described by Prasad (1991a; 1991b) and various clinical manifestations such as short stature, delayed sexual maturity, hepatosplenomegaly and delayed bone age reported by Korman (1990).

Harmful effect of geophagia in pregnancy has been demonstrated in a large number of studies related to the microbial and parasite infections such as helminthes and the presence in the soil of heavy metals such as arsenic, lead and cadmium (Hunter, 1973; Horner et al., 1991; Corbett et al., 2003; Wigle et al., 2008; Al-Rmalli et al., 2010; Baidoo et al., 2010; Nyanza et al., 2014). One case of fatal soil peritonitis in an African women suffering from geophagia has been reported (Woywodt and Kiss, 2000). This peritonitis is due to the perforation of sigmoid colon. Frequent consumption of clay during pregnancy has been considered as habit rather than physiological need for the cessation of undesirable manifestations such as nausea, vomiting occurring in the first trimester of pregnancy (Horner et al., 1991).

Numerous studies have shown that pre-eclampsia characterized by a gestational hypertension associated with pathological edema, proteinuria, coagulation abnormalities, reduced utero-placental blood flow, and intrauterine growth restriction, is one of the maternal complications of pregnancy which can result in fetal or infant death. Obesity has been indeed strongly associated with high risk of pre-eclampsia (Sibai et al., 1995). However, other physiopathological features such as insulin resistance, increased lipid availability, higher cholesterol and triglyceride levels may also involve in increased risk for early pregnancy loss (de Weerd et al., 2003). Shed membranes particules of leucocytes (Meziani et al., 2006), oxidative stress (Hubel, 1999), elevated plasma lipids (Lorentzen and Henriksen, 1998) and activated neutrophils (Clark et al., 1998) are among factors inducing in endothelial cell activation during pre-eclamptic pregnancy. Under these conditions, placenta is under oxidative stress with increased production of lipoperoxides and decreased antioxidant protection (Walsh, 1998; Wang and Walsh, 2001). The subsequent release of ROS/RNS, tumor necrosis and myeloperoxidase by neutrophils will lead into the smooth muscle inflammation in pre-eclampsia.

Despite its absorbent properties in detoxifying phytotoxin, lowering the incidence of nausea, natural clay still remains toxic for human consumption in normal and long term conditions. In the absence of evidence implicating clay minerals or micronutrients in pre-eclampsia process, we have postulated that geophagia could exacerbate this pregnancy complication through reactive oxygen/nitrogen species (ROS/RNS) generated from activated macrophages. In addition, the release of free iron from ferritin during inflammatory process producing cytokines could be the cause of the production of hydroxyl radical. Haber-Weiss and Fenton reactions involving iron as catalytic trace element do generate this OH radical (Halliwell and Gutteridge, 1990; Blakely et al., 1990).

Haber-Weiss reaction: $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + {}^{\bullet}OH + OH^{-}$

Fenton-type chemistry: ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3} + {}^{\bullet}OH + OH^{-}$

Natural clay contains iron among other minerals. Chronic feeding of iron has been associated indeed with an increased free radical generation in the colon and increase

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Abbreviations: CAT, Catalase; **Fe,** iron; **FOX,** ferrous xylenol orange; **H_2O_2,** hydrogen peroxide; **SOD,** superoxide dismutase; **GPx,** glutathione peroxidise; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione; **GRx,** glutathione reductase; **TAS,** total antioxidant status; **Hb,** haemoglobin; **LOO^-,** lipid radical; **$LOO^{\bullet}$;** alkoxyl radical; **LOOH,** lipid hydroperoxides; **LPO,** lipid peroxide; **MDA,** malondialdehyde; **NADH,** nicotinamide adenine dinucleotide reduced form; **$O_2^{\bullet -}$,** superoxide anion; **$^{\bullet}OH$,** hydroxyl radical; **8-OHdG,** 8-hydroxy-deoxyguanosine; **ROS,** reactive oxygen species; **RNS,** reactive nitrogen species; **MSR,** methionine sulphoxide reductase; **AST,** aspartate aminotrnasferase; **ALT,** alanine aminotransferase; **ABTS,** 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate]; **ORAC**, oxygen radical absorbance capacity; **Cox-2,** cyclo-oxygenase, **Nf-kB,** nuclear factor kappa B; **HELLP**, haemolysis, elevated liver enzymes, low platelet count; **WHO**, World Health Organization.

lipid peroxidation and thus constitute a risk factor for colorectal cancer (Lund et al., 2001).

As a transition element, ionic form of iron participates in one-electron transfer reactions and plays an important role as a prosthetic group in enzymes that catalyze redox reactions. Unfortunately this capacity enables iron to participate in the above mentioned reactions such as "Fenton reaction" generating more potent secondary ROS. The extent of $^{\bullet}OH$ formation is largely determined by the availability and location of the metal ion catalyst. The availability of free Fe is controlled in physiological systems. Fe that is not incorporated into iron-utilizing proteins is rendered largely unavailable for participation in free-radical reactions by sequestration in storage (ferritin) or transport proteins (transferrin).

Other by-products of lipid peroxidation such as malondialdehyde (MDA) (Marnett, 1999) and M_1G deoxyribose resulting from the combination of MDA and deoxyribonucleoside (Marnett, 2000) together with 8-hydroxy-2'-deoxyguanosine (8-OHdG) a major DNA damage metabolite (Wong et al., 2003; Guetens et al., 2002; Shigenaga et al., 1994) by free radicals may also be involved in inflammatory process increasing NF-kB activation which will enhance COX-2 induction. Increase activity of this enzyme will indeed affect the production of isoprostanes formed from the peroxidation of arachidonic acid (Liu, 1999).

The question of biological association of lipid peroxidation and pre-eclampsia has been discussed by Gupta et al., (2009). Placental oxidative stress is involved in the etiopathogenesis of pre-eclampsia as a result of ischemic reperfusion injury. Intermittent and inadequate placental perfusion in the spiral arteries as a result of repeated hypoxia/reoxygenation insult may indeed lead to the activation of xanthine oxidase which is a source of superoxide anion (Many, 2000; Raijmakers, 2004). This ischemic reperfusion injury constitutes a promoter of lipid peroxidation and endothelial cell dysfunction observed in pre-eclampsia.

Fortunately, in healthy subjects or non-pregnant women, the extent of oxidative stress is minimized by a strong defense line of antioxidants against ROS/RNS. While superoxide dismutase (SOD) converts two superoxide anions ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2), catalase (CAT) and glutathione peroxidase (GPx) convert H_2O_2 to oxygen and water. Vitamins C, E and A remove the newly formed free radicals before they can initiate chain reactions. A repair cell structures damaged by free radical attack are done by DNA repair enzymes and methionine sulphoxide reductase (MSR).

Apart from vitamins A and C supplementation which showed 54% reduction in the risk of developing pre-eclampsia, other supplementation based on iron (Mahomed, 1998) and a mixture of iron and folate (Mahomed and Hytten, 1989), magnesium (Makrides and Crowther, 2001), fish oil (Makrides et al., 2006) did not prevent this gestational hypertension.

In contrast, only calcium supplementation has reduced the incidence of both pre-eclampsia and hypertension (Villar et al., 2003). Taking together the above considerations, we suggest that the subsequent activation of the nuclear factor NF-kB by ROS/RNS generated from activated macrophages and activation of xanthine oxidase stimulates the induction of COX-2. This enzymatic induction leading to the expression of inflammatory cytokines could be one of the molecular mechanism underlying pre-eclamptic conditions. Supplementation of zinc or eating clay containing zinc could overcome such pregnancy complication.

MATERIALS AND METHODS

Human subjects

A total of 117 women were enrolled in this study: 32 controls (non-pregnant), 42 pregnant non-eclamptic and 45 pregnant pre-eclamptic (30-34 week gestation).

Selection criteria

Inclusion criteria

Control: non-pregnant women (20-35 years old) without any sign of hypertension, diabetes or viral infection. They were recruited from student enrolled at an academic Institution (Medical University of Southern Africa)

Pregnant pre-eclamptic and non-eclamptic women were recruited from consulting room at tertiary hospital (George Mukhari Hospital). For pre-eclamptic group, the following symptoms have been observed and noted: headache, upper abdominal pain, visual disturbance, hypertension (high blood pressure, proteinuria). More importantly, the consumption of soil and the location has been also recorded.

Exclusion criteria

All subjects having infectious diseases such as HIV, Hepatitis were excluded from this study. In addition, control subjects did not have hypertension or renal diseases not suffering from any other diseases affecting oxidative stress parameters such as cardiovascular diseases, anemia, family hypercholesterolemia, diabetes and cancer. Pre-eclamptic pregnant women taking iron for anemia were excluded from this study.

Animal subjects

Ninety (90) pregnant Wistar albino (*rattus Norvegicus*) rats (of initial weight 200 ± 5 g) were fed (*ad libitum*) a standard chow diet (AIN-93G Formulation) according the following dietary regimen:

Group 1 (control group) did not receive any supplementation.
Groups 2 and 3 (Fe gp2 and Fe gp3) were fed with a high iron diet (the diet was supplemented with 2.5% pentacarbonyl iron (CI-98.0% purity) according to the protocol of Plummer et al. (1997).
Group 3 (Fe + vitamins C and E) received iron plus vitamins C and E supplementation: 1000 mg vitamin C per kg diet (ascorbic acid) and 500 mg vitamin E per kg diet ≡ 750 IU α-tocopherol (10x RDA)

(Plummer et al., 1997). At 12 month, the 2.5% CI was replaced by 0.5% dicyclopentadienyl iron $[Fe(C_5H_5)_2]$ (CAS-102-54-5) (because of poor iron loading). The rats received human care in accordance with the guidelines of the Animal Ethics Committee of the University. They were studied for 24 months. Five rats from each group were sacrificed every four months up until 24 months for blood sampling. These samples were used for oxidative stress and transaminases evaluation.

Materials and chemicals

All chemicals, Vitamins and Kits for iron and ORAC evaluation were purchased from Sigma Company (Munich, Germany). For other tests, we used kits from Randox Laboratories, UK and Roche Diagnostics (Indianapolis, IN).

Analytical methods

Apart from selenium, vitamins A and E, all procedures using kits commercially available were followed according to the manufacturer's instructions.

i) Iron was evaluated by potentiostatic coulometry using a kit commercially available from Sigma Company. This method has been adapted by Dorner et al. (1981) to measure iron in small sample volume.

ii) Lipoperoxide (LPO) concentrations were measured spectrophotometrically at 560 nm using ferrous oxidation with xylenol orange (FOX II) assay according to the method of Nourooz-Zadeh et al. (1994) based on the principle of rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions.

iii) 8 hydroxyl deoxy-guanosine (8-OHdG): Plasma levels of 8-OHdG were evaluated at 450 nm on a microplate plate reader using a commercial kit from the Japan Institute for the Control of Aging (Fukuroi, Japan). The method is based on a competitive *in-vitro* enzyme-linked immunosorbent assay for quantitative measurement of this DNA metabolite in tissue, serum and plasma (Toyokuni et al., 1997 and 1999).

iv) Superoxide dismutase (SOD): Erythrocyte SOD activity was determined by the method of Arthur and Boyne (1985) using a commercial kit obtained from Randox Laboratories, UK. This method uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured spectrophotometrically at 505 nm by the degree of inhibition of this reaction.

v) Glutathione peroxidase (GPx): The determination of erythrocyte GPx activity was based on modification of the method of Paglia and Valentine (1967) using a commercial kit obtained from Randox Laboratories, UK. This method involves the oxidation of glutathione (GSH) by cumene hydroperoxide catalyzed by GPx. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$ and the decrease in absorbance is then measured spectrophotometrically at 340 nm.

vi) Transaminases: Aspartate aminotransferase (AST) and alanine aminotranferase (ALT) activities were evaluated using an automate auto-analyser (Cobas Interga 400, Holliston, MA) and kits from Roche Diagnostics (Indianapolis, IN), using the spectrophotometric method of Karmen (1955) in which malic acid dehydrogenase is added to convert the oxaloacetic acid formed by AST to malic acid with the simultaneous oxidation of the coenzyme $NADH_2$ (reduced nicotinamide adenine dinucleotide) to NAD (nicotinamideadenine dinucleotide).

In the case of ALT, lactic acid dehydrogenase is used and lactic acid is formed simultaneously with NAD. The decrease in absorbance of $NADH_2$ as it is oxidized to NAD is followed at 340 nm by the spectrophotometer in each case.

vii) Oxygen radical absorbance capacity (ORAC): Liver total antioxidants were measured using oxygen radical absorbance capacity (ORAC) system which is made up of B-phytocoerythrin as a fluorescent indicator protein, 2-2'-azo-bis(2-aminodinpropane) dihydrochloride a peroxy radical generator, and the water-soluble vitamin E analogue Trolox (Aldrich-Sigma, Munich, Germany) as a reference standard. The total antioxidant capacity was expressed as ORAC units, where one unit equals the net protection by 1 µmol Trolox/l (Cao and Prior, 1999).

viii) Total antioxidant status (TAS): Total antioxidant status (TAS) in heparinised plasma was determined according to Miller et al. (1993) method using a commercial kit obtained from Randox Laboratories Limited, U.K. In this method 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate] ($ABTS^{(R)}$) is incubated with a peroxidase (metmyoglobin)and hydrogen peroxide ($H_2O_{2)}$ to produce the radical cation $ABTS^{(R)\cdot+}$ measured at 600 nm.

ix) Selenium: Selenium in plasma was estimated by the method of Pleban et al. (1982).

x) Vitamin C (ascorbic acid) was estimated spectrophotometrically at wavelength of 700 nm in plasma by acid phosphotungstate method of Aye (1978).

xi) Vitamin E (α -tocopherol): Vitamin E (α -tocopherol) in plasma was extracted using xylene, and its level was estimated spectrophotometrically at 520 nm according to the method of Baker and Frank (1968).

Statistical analyses

All data were presented as mean ± standard deviation (SD) for each biomarker. The SAS statistical package was used. Statistical analysis was performed by independent Student's *t*-test. A probability value of $p<0.05$ was considered statistically significant.

RESULTS

Experimental animals

The data obtained from pregnant Wistar albino (*rattus Norvegicus*) rats fed high iron diet with and without vitamin supplementation demonstrated that vit C and E reduced for a limited period of time (20 months) the extent of oxidative stress as shown by the increased activity of SOD and GPx with subsequent decrease of LPO and 8-OHdG levels (Table 2). Hepatic enzymatic activities of transferases ALT and AST are also increased respectively by 29 and 34%. It has been noted that over a prolonged period, supplementation of high doses of vitamins C and E did not reduce the adverse effect of ROS/RNS and the by-products of lipid peroxidation and attenuate the subsequent oxidative DNA damage leading to cancer.

DISCUSSION

Several trials failed to establish links between pre-eclampsia

Table 1. Biomarkers of oxidative stress in pregnancy and pre-eclampsia in humans

Parameter	Control	Normal pregnant women	Pre-eclamptic women
LPO (µmol/L)	32.71 ± 5.84	55.45 ± 9.07	68.19 ± 6.44
8-OHdG (ng/ml)	33.52 ± 4.61	48.34 ± 8.45	50.24 ± 5.96
SOD (U/g Hb)	4394.51 ±1152.33	4134.12 ± 532.67	4005 ± 1037.28
GPx (U/g Hb)	1173.49 ± 234.75	970.64 ± 145.34	
TAS (mmol/L)	1.45 ± 0.18	1.23 ± 0.12	0.62 ± 0.14
Vitamin C(mg/dl)	0.12 ± 0.06	0.10 ± 0.05	0.07 ± 0.03
Vitamin E (mg/dl)	1.13 ± 0.07	1.09 ± 0.35	0.95 ± 024
Selenium (µmol/L)	0.85 ± 0.24	0.76 ± 0.20	0.38 ± 0.15

Table 2. Biomarkers of Oxidative stress in iron overloaded animal (rats0 model at 20 month

Group	Fe (µmol/L)	ALT (IU/L)	AST (IU/L)	SOD (U/g Hb)	GPX (U/gHb)	ORAC(mM Trolox)	8-OHdG (nmol/ml)
Group 1 (control)	62.5 ± 6.2	63.2 ± 5.3	130 ± 4.6	2165 ± 444	214 ± 11.9	14.2 ± 2.8	123 ± 21
Group 2 (Fe)	122.0 ± 10.5	329 ± 48.6	202.2 ± 50.5	1584 ± 360	179.1 ± 16.1	7.5 ± 1.4	455 ± 151
Group 3 (Fe + Vit C & E Supplementation)	119.1 ± 9.2	234.6 ± 48.3	134.6 ± 48.6	1626 ± 199	228.8 ± 17.6	13.0 ± 2.5	346 ± 117

and the following interventions: balanced protein-energy supplementation (Kramer, 2000), isocaloric balance protein supplementation (Kramer and Kakuma, 2003), and protein and energy restriction for obese women (Kramer, 2007) despite the relationship between pre-eclampsia and obesity.

It has been reported that calcium reduce the risk of gestational hypertension (Villar et al., 2003) in contrast with the non-significant effect of calcium supplementation observed in the largest trial conducted by the National Institute of Health-USA. These negative observations have been made for iron and folate supplementation as above mentioned. There is no biological link either between magnesium, fish oil and gestational hypertension (Altman et al., 2002; Duley, 2009).

Fortunately, prophylactic interventions with vitamins E and C supplementation were associated with 54% reduction in the risk of developing pre-eclampsia. Among various WHO recommendations (WHO, 2011a; 2011b) for prevention and treatment of pre-eclampsia and eclampsia, the high quality of evidence has been observed only for magnesium sulfate (Duley et al., 2010a, 2010b), vitamins C and E administration (Rumbold et al., 2008) which make them to be strongly recommended (WHO, 2010, 2011). Calcium (Hofmeyr et al., 2010) and vit D supplementation and corticoids administration especially for the treatment of the "haemolysis elevated liver enzymes low platelet count" (HELLP) can help (Woudstra et al., 2010). However these supplementation programmes should be monitored and evaluated for their effects.

Usually labour induction and administration of antihypertensive drugs (Duley et al., 2006; Abalos et al., 2007) and diuretics (Churchill et al., 2007) for pre-eclampsia or gestational hypertension at term are recommended. However, the benefit and potential harm of these strategies need to be investigated especially in rural African settings where accurate gestational age assessment is difficult to be conducted due to the late initiation or absence of antenatal care.

Data from our studies, confirmed those reported by Gupta et al., (2009) on lipid peroxides and antioxidants status in pre-eclampsia. As shown in Table 1, biomarkers of oxidative stress (LPO, 8-OHdG) increased while enzymatic activities (SOD, GPx) decreased more during pre-eclampsia than the normal pregnancy suggesting the involvement of ROS/RNS from the activation of macrophages.

The decrease of GPx activity, a seleno-enzyme, observed in iron overload rats (Table 2), may justify the reduction of this micronutrient: selenium being used in biosynthesis of this enzyme in humans and animals.

In contrast, TAS, vitamins C and E were reduced suggesting their role in scavenging free radicals and especially OH radical generated from Haber-Weiss and Fenton reactions and from the activation of macrophages. Those reactions take place because of the release of free iron from ferritin during inflammatory process producing cytokines (Casanueva and Viteri, 2003). Excess iron intake through geophagia could induce toxic reactions (Rush, 2000). The resulting high ferritin level will lead into the failure of maternal plasma

expansion (Silver et al., 1998).

Our results have confirmed those of Scholl (2004, unpublished data) on the association between increased iron stores and the excretion of 8-OHdG, a marker of oxidative damage to DNA and may influence the long-term health outcomes of infants after birth by inducing G→T mutations during DNA replication which increase the risk of cancer (Satoko et al., 2012; Shibutani et al., 1991).

Our experimental animal model has indeed demonstrated in iron overload conditions, a decrease of total antioxidants and an increase of 8-OHdG in plasma as well as in rat hepatocytes; antioxidative enzymatic activities SOD and GPx being reduced by 26.9 and 16.4%, respectively. Supplementation in vit C and E can help to reduce the extent of oxidative stress: 2% for SOD and 27% for GPx. It can also alleviate maternal plasma expansion. Inadequate plasma volume expansion as depicted by the reduction of creatinin, uric acid and hematocrit has been indeed observed in women with pre-eclampsia (Silver et al., 1998).

However, high doses of vitamins ((Cheng, 1999; Muller, 2010) and antioxidants (Bjelakovic et al., 2007) supplementations can increase the mortality rate in randomized trials. Generally, food and to lesser extent, soil contains less heme iron than nonheme iron. The lower absorption rate iron containing soil may be increased depending on the level of iron stores (Monsen, 1988). Iron deficiency observed in many African pregnant women may thus trigger the absorption rate of this form of heme. Iron and zinc deficiencies usually associated with plant-based diets in poor countries are not associated with vegetarian diets in wealthier countries (Janet, 2003). The question of bioavailability of iron, zinc, and other trace minerals in pregnant women is not completely resolved and depends on factors such as gastric acidity, presence of inhibitors (Hurrell et al., 1999; Hunt, 2003). The relative bioavailability of iron compounds is determined by their solubility in stomach's gastric juice. Iron deficiency can be prevented using food fortification by addition of enhancers such as ethylen-diamine-tetraacetic acid, glycine, ascorbic acid (Hurrell, 1997). Hilt et al. (2011) have even demonstrated the improvement of iron solubility in dilute acid when Magnesium and Calcium have been incorporated into the nanostructure of Fe_2O_3. Nevertheless, in acidic and decrease of iron stores conditions, and in absence of inhibitors, nonheme iron can thus play a role as catalyst metal in Haber-Weiss and Fenton reaction producing more OH radical which will damage cells.

While the above mentioned clay minerals do not have apparent toxicity, iron may be harmful in pre-eclamptic/eclamptic women because of generation of ROS/RNS inducing through NF-kappa B. the expression of inflammatory genes. This gene expression is susceptible to exacerbate the gestational hypertension in short term and cancer in long term process; inflammation being the intermediate step before the occurrence of pre-neoplastic phase.

In contrast, zinc supplementation needs to be taken into consideration because of its role in the metabolism of several protein enzymes. Deficiency in this important trace element can indeed affect many functions including the induction of pre-eclampsia. It also plays an important role in cell-mediated immune functions as anti-inflammatory and antioxidant agent (Prasad, 2004).

Conclusion

Due to its ubiquitous properties, eating soil containing iron may be harmful for pregnant and mostly pre-eclamptic/eclamptic women where endothelial dysfunction can occur as a result of activation of macrophages. As molecular mechanism, ROS/RNS resulting from activated macrophages will trigger the binding of AP-1 to NF-kB for the induction of COX-2 leading to the expression of inflammatory genes responsible for clinical manifestations of pre-eclampsia.

Soil containing magnesium and zinc should be beneficial to pregnant as well as pre-eclamptic/eclamptic women. In the particular case of zinc, this trace element will stimulate the upregulation of mRNA and DNA-specific binding for A20. This transactivating factor will then inhibit the activation of NF-kB leading to the down regulation of inflammatory cytokines (Prasad et al., 2004, 2011; Bao et al., 2010).

Conflict of Interests

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

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Full Length Research Paper

Detection of nDNA antibodies in rheumatoid arthritis patients by an immunofluorescent technique

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The main objective of this study was to focus on the relationship of nDNA antibodies with rheumatoid arthritis (RA) and to determine the specificity, sensitivity, positive predictive value and negative predictive value of nDNA for the clinical diagnosis of rheumatoid arthritis. The study included a total of 40 rheumatoid arthritis cases that fulfilled the American College of Rheumatology (ACR) diagnosis criteria for rheumatoid arthritis as well as 40 age and sex matched controls. Agglutination technique was used for qualitative and semi-quantitative measure of rheumatoid factor (RF) and indirect immunofluorescence assay was employed for the determination of anti-nDNA antibodies. RF latex agglutination test was carried out to confirm RA cases, out of which four (10%) turned out to be negative, so only 36 RA cases were further investigated and analyzed through indirect immunofluorescence assay. Out of 36 individuals, 31 (86%) were negative, three (8.3%) were strong positive and two (5.5%) were weak positive. No significant association was found between nDNA antibodies and rheumatoid arthritis disease.

Key words: Rheumatoid arthritis, anti-native DNA antibodies, immunofluorescence assay, sensitivity, positive predictive value, negative predictive value.

INTRODUCTION

Joint disorders involving inflammation of one or more joints are collectively called arthritis. These conditions are classified as over hundred different types. Most important types of arthritis are immune-mediated, for example psoriatic arthritis and rheumatoid arthritis (RA). The word “rheumatoid” originated from Greek rheuma meaning "flow or current" and -oid "resembling" that translates as joint inflammation that is similar to rheumatic fever. It is also known as chronic inflammatory polyarthritis. It is a progressive and degenerative autoimmune disorder in which defense mechanism of the body starts to invade the articular system of the body. In this long-term disease, the joints become inflamed and swelled, resulting in pain and stiffness throughout the body. This disease can lead to chronic inflammation and irreversible destruction of bone and cartilage. It can also contribute to the occurrence of clinically significant severe comorbid conditions if not properly treated (Kalreskog et al., 2009). The risk of mortality for patients with rheumatoid arthritis is 38% greater than the general population throughout

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Abbreviations: RA, Rheumatoid arthritis; **ACR,** American College of Rheumatology; **RF**, rheumatoid factor; **IFA,** immunofluorescence assay.

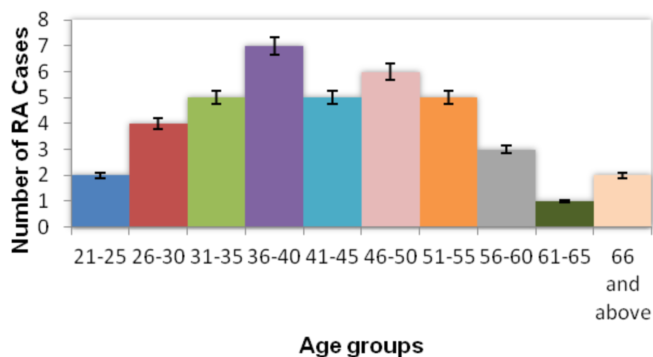

Figure 1. Age distribution in RA cases.

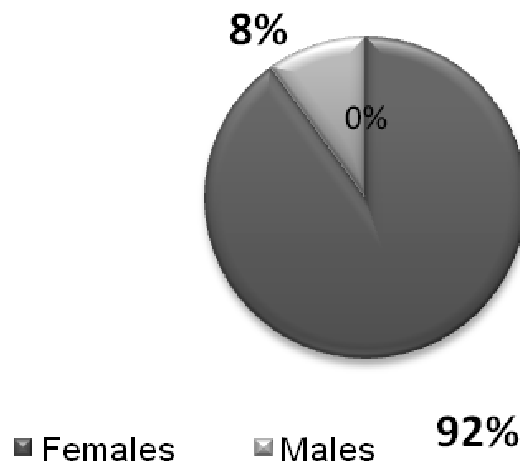

Figure 2. Gender ratio in RA case.

the world whereas this ratio for female patients is 55% more than the general population (Gabriel et al., 2003). Male gender, rheumatoid factor, extra-articular manifestations and co-morbidity have been implicated as major predictors for increased mortality (Rochmis et al., 1974).

Until 1973, no study was done to determine the prevalence of antibody to nDNA in a large group of patients with well-characterized rheumatoid arthritis. After decades of development in the field of laboratory techniques of diagnostics, more accurate and specific methods are now available. In the urban population of Southern Pakistan, the prevalence of RA is reported to be 0.142 per 100 inhabitants, whereas in northern Pakistan the estimated prevalence is 0.55 per 100 inhabitants (Hameed and Gibson, 1996; Farooqi and Gibson, 1998). According to a local survey, female to male ratio in Pakistani RA patients is 4:1 (Alam and Kidwai, 2011). This is the first study done for the detection of nDNA antibodies in the sera of rheumatoid arthritis patients by indirect immunofluoresce assay. The study focused on the relationship of nDNA antibodies with rheumatoid arthritis and to determine the specificity, sensitivity, positive predictive value and negative predictive value of nDNA for the clinical diagnosis of rheumatoid arthritis.

MATERIALS AND METHODS

The study included a total of 40 rheumatoid arthritis cases that fulfilled at least four of the seven the American College of Rheumatology (ACR) criteria. A total of 40 individuals were included in this study on initial clinical diagnosis as RA patients. Also, 40 healthy individuals that were age and gender matched with RA cases were included as controls. There were two (5%) RA cases from age group 21 to 25, four (10%) from age group 26 to 30, five (12.5%) from age group 31 to 35, seven (17.5%) from age group 36 to 40, five (12.5%) from age group 41 to 45, six (15%) from age group 46 to 50, five (12.5%) from age group 51 to 55, three (7.5%) from age group 56 to 60, one (2.5%) from age group 61 to 65 and two (5%) from age group 66 and above (Figure 1). Around 92% of the RA cases were female (Figure 2). Therefore, the female to male ratio was approximately (11:1). Average age of cases was 40.9 years.

Agglutination technique was used for qualitative and semi-quantitative measure of rheumatoid factor (RF) and indirect immunofluorescence assay was employed for the determination of anti-nDNA antibodies. For serum agglutination RF test, RF latex kit was used by Spectrum, an Egyptian Company for Biotechnology (catalog number: 518 002). It is the rapid latex agglutination test for the qualitative screening and semi-quantitative determination of RF in human serum. This test was employed to confirm RA cases included in this study. For indirect Immunofluorescence assay, anti-nDNA antibodies kit was used for the determination of anti-nDNA antibodies *in vitro* by Orgentec (catalog number: ORG 801). Statistical analysis like univariate analysis, Chi-square test and Fisher's exact test were carried out by SPSS (ver. 13). Specificity, sensitivity, negative predictor value and positive predictor value were calculated by using online software "Diagnostic Test Statistics" (Maceneaney and Malone, 2000).

RESULTS

Different parameters in this study were calculated and for further analysis of the findings, graphical formats were used. Pain levels were estimated in each RA case from clinical analysis. Patients were asked to score for their pain level from one to ten, according to the intensity of the pain. These scores were calculated into percentages. According to the standard in Figure 3, 10 levels of pain intensity were established in which 0 stands for no pain in the joints, whereas, 100 stands for severe pain intensity. Level 3 had one (2.5%) number of cases, level 4 had four (10%), level 5 had six (15%), level 6 had eight (20%), level 7 had 15 (37.5%) and level 8 had six (15%) number of RA cases (Table 1). RF latex agglutination test was carried out to confirm RA cases, out of which four (10%) turned out to be negative (Figure 4). As RF test is one of the important criteria in ACR guidelines, the negative cases of RF latex agglutination test were excluded from the study to ensure further standard results of indirect immunofluorescence assay (IFA). Semi-quantitative RF latex test was also carried out to estimate the titer of RF in blood samples of rheumatoid arthritis patients.

After confirmation through RF latex agglutination test, only 36 RA cases were further investigated and analyzed through indirect immunofluorescence assay. Out of 36

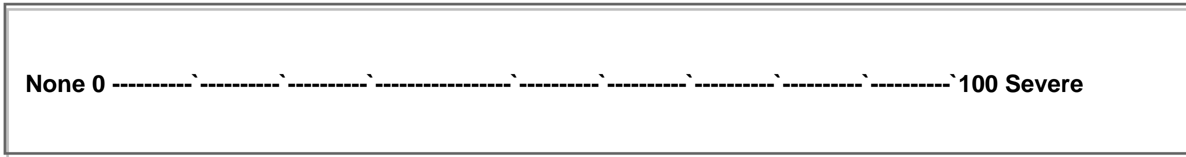

Figure 3. Standard used for the calculation of pain intensity.

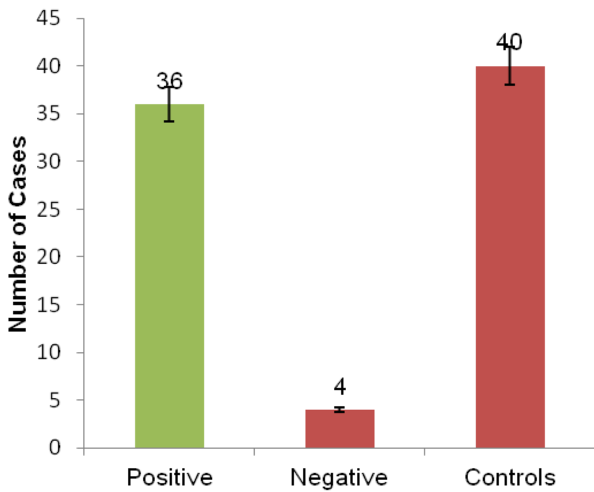

Figure 4. Bivariates of RF in RA patients (n = 40) and control groups (n = 40).

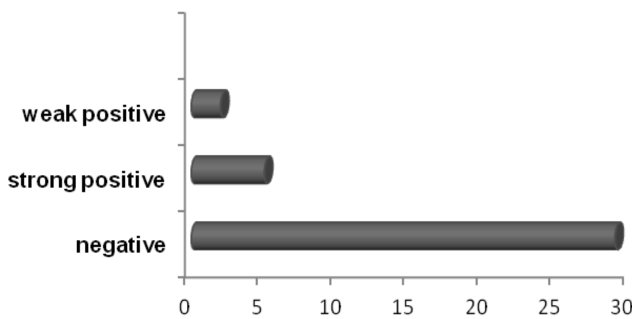

Figure 5. IFA for nDNA antibodies results.

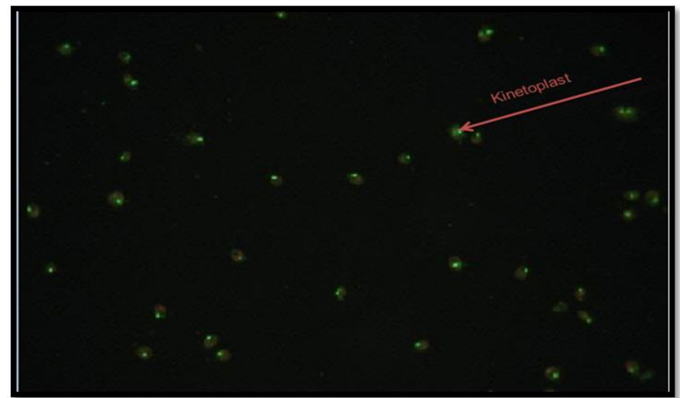

Figure 6. IFA for nDNA antibodies positive result (100× magnification power).

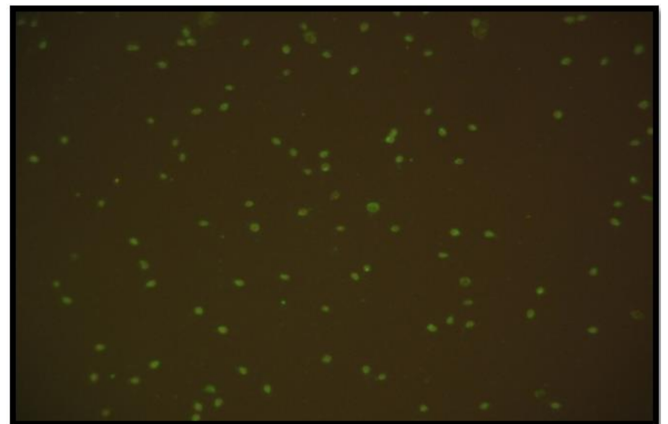

Figure 7. IFA negative result (100× magnification power).

individuals, 31 (86%) were negative, three (8.3%) were strong positive and two (5.5%) were weak positive (Figure 5). The slides used in IFA for nDNA antibodies kit contained hemoflagellates Crithidia luciliae. In these pear-shaped microorganisms, the helical nDNA is present in the large mitochondrion, part of which is called kinetoplast. It appears to be slightly concave disc-shaped structure found between the central nucleus and the basal body of the flagellum. In positive samples,
formation of a stable three-part complex consisting of fluorescent antibody bound to human anti-nDNA antibody, which is bound to nDNA antigen (Figure 6). This binding was visualized using fluorescent microscope with required filters, under which the kinetoplast appears to be of bright apple green color. While in negative samples, kinetoplast showed no fluorescence (Figure 7).

Statistical analysis

Mean and standard deviations were also calculated for both techniques (Table 2). Mean values for RF test was 38.2 ± 2 for positive results whereas for negative results, it was 22 ± 18. Mean values for IFA for nDNA antibodies for positive results was 20.5 ± 15.5 whereas for negative results, was 33.5 ± 2.5. A number of statistical analysis measures were applied (Table 3). Chi-square test and

Table 1. Pain intensity in RA cases.

Pain level	Pain level in percentage (%)	Number of RA cases	RA cases in percentage (%)
1	10	0	0
2	20	0	0
3	30	1	2.5
4	40	4	10
5	50	6	15
6	60	8	20
7	70	15	37.5
8	80	6	15
9	90	0	0
10	100	0	0

Table 2. Univariant analysis of RF and IFA test results.

Analysis applied	Mean	
	Positive	Negative
RF	38.2±2	22±18
IFA	20.5±15.5	33.5±2.5

Table 3. Statistical analysis of RF test results and IFA for nDNA antibodies test results.

Status	Case	Control	Fisher's exact test value (p)	Chi square test value (p)
RF				
Positive	36	0		
Negative	4	40	*<0.0001	*<0.0001
Total	40	40		
IFA test				
Positive	5	0		
Negative	31	36	**0.0539	**0.0637
Total	36	36		

*Extremely statistically significant, ** not statistically significant.

Table 4. Sensitivity, specificity, positive and negative predictor value of nDNA for the clinical diagnosis of RA.

Antibody	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%)	NPV (%)
nDNA	13.88 (4-29)	100 (90-100)	100 (47-100)	43.73 (41-66)

PPV, Positive predictive value; NPV, negative predictive value; CI, confidence interval.

Fisher's exact tests were employed to determine association between nDNA antibodies and rheumatoid arthritis. As Chi-square test is used for larger population whereas Fisher's exact test is used for small number of population, both tests were used for comparison of statistical analysis.

Both tests showed that there was no significant association of nDNA with RA, while RF was found to be significantly associated with RA. In this study, the IFA nDNA antibodies test was 100% specific and 13.88% sensitive for rheumatoid arthritis. Table 4 represents specificity, sensitivity, negative predictive value and positive predictive value calculated by diagnostic test statistics tool for the clinical diagnosis of rheumatoid arthritis. Furthermore, 95% confidence interval was calculated by binomial expansion.

DISCUSSION

Rheumatoid arthritis is a disease caused by dysfunctional immune system of the human body. Throughout the

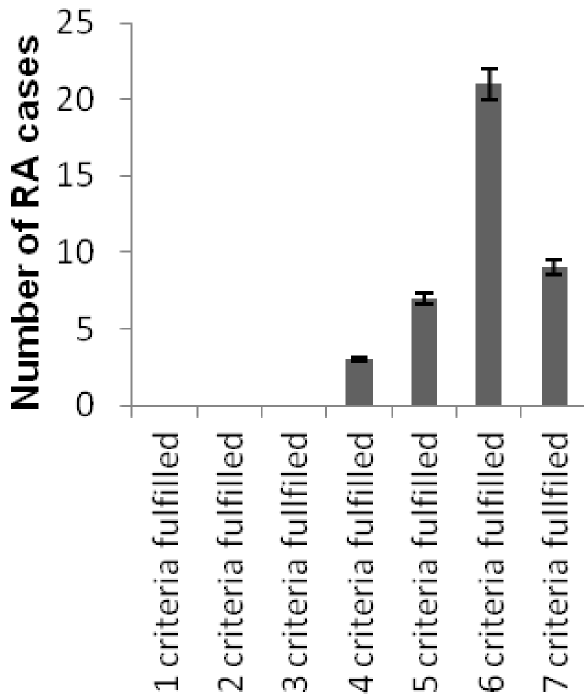

Figure 8. ACR criteria for diagnosis agreement in RA cases (n = 40).

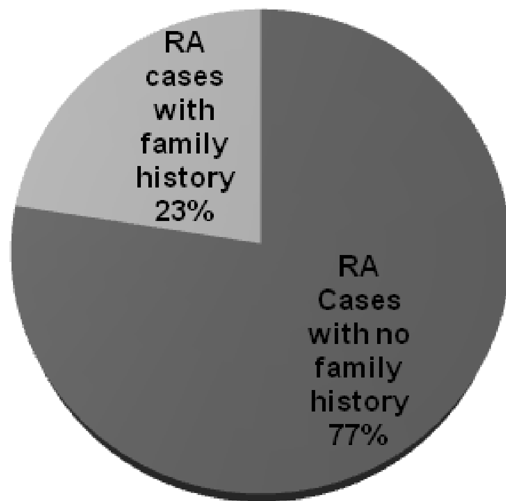

Figure 9. Family history in the studied RA cases.

history of medical advancements and discoveries, Rheumatology has remained a well sought field. Unfortunately, patients with rheumatoid arthritis disease have not experienced significant improvements in survival over the past four decades, despite dramatic downfall in the overall rates of mortality in the general population (Gonzalez et al., 2007). The present study was carried out for the evaluation of diagnostic measures of rheumatoid arthritis in routine laboratories. To establish this, two immunology-based techniques were employed and a number of other factors were studied among rheumatoid arthritis patients. Guidelines and criteria from ACR were considered to diagnose and select RA population from general population. Only three (7.5%) fulfilled four criteria out of seven, seven (17.5%) fulfilled five criteria, 21 (51%) fulfilled six criteria and nine (22.5%) fulfilled all seven criteria (Figure 8). The female to male ratio was 11:1, indicating higher number of females in RA cases. There can be several reasons for the unequal gender distribution in rheumatoid arthritis. Hormonal exposures, reproductive factors and live birth histories may have a contributing role in greater female ratio among RA cases. The distribution of RA cases in age groups was totally random and can be considered as the representative RA cases among general population of the same age groups. The distribution of age groups of RA cases indicate that the highest number of RA cases belonged to the age group 31 to 45 years (40%) whereas age 21 to 25 and above 65 had lowest number of RA cases (5%).

According to Orozco et al. (2007), there is a strong genetic linkage of rheumatoid arthritis among populations throughout the world. The pattern of rheumatoid arthritis hereditary is not simple, as there can be several genetic factors involved, that is the polygenic inheritance pattern (Deighton and Walker, 1991).

No significant and well established study has been carried out in Pakistan to investigate genetics of RA in this region. For this reason, the genetic linkage was determined by clinical history of each RA case. There were six RA cases (23%) out of 40, who had family history of rheumatoid arthritis disease (Figure 9). All of them were females and five out of those six cases might have inherited rheumatoid arthritis disease from their mothers.

For the estimation of the overall disease status and condition of RA cases, a number of clinical parameters were considered. This was done to estimate the disease prognosis of each RA case. Edwards et al. (2009) suggested that pain is the most common and most impairing stressing factor in rheumatoid arthritis patients. This is the reason pain intensity levels were estimated. The highest was level 7, which indicates 37.5% of RA patients suffer from very intense pain, thus have severe conditions. This data helps in determination of severity and stage of RA disease, and can also be used for monitoring the treatment therapy effectiveness.

Recently, Salaffi et al. (2010) reported that overall health is significantly affected in RA patients. Health conditions are important hallmarks of rheumatoid arthritis disease. During clinical analysis of RA cases, overall health was observed and recorded through a series of questions (Figure 10). Patients were given four health statuses among which they were asked to mark their current health condition. There were 16 (40%) out of 40

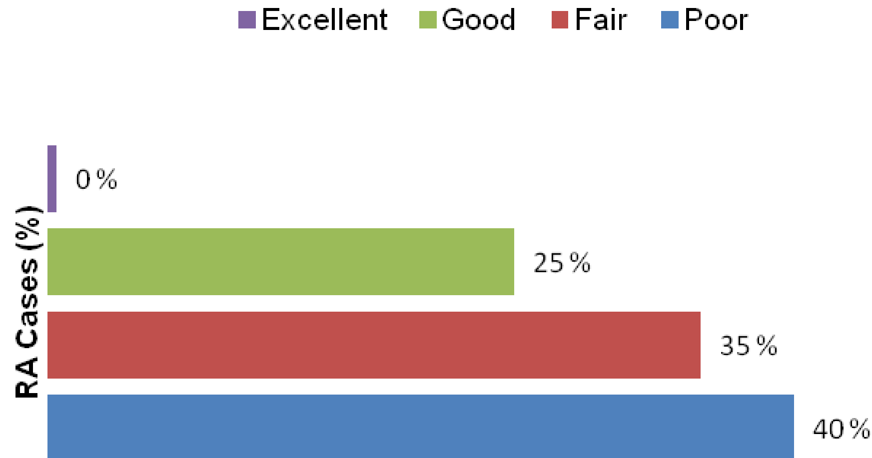

Figure 10. Overall health of RA cases.

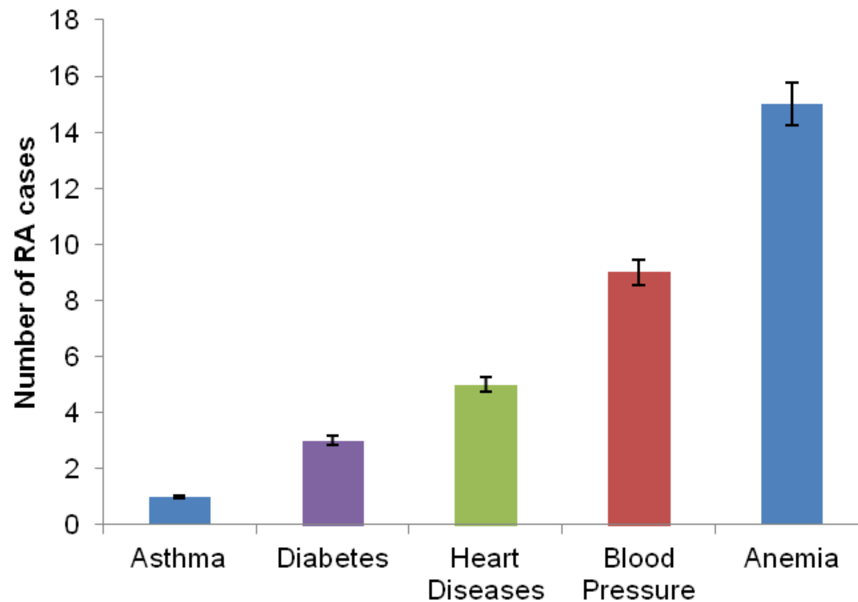

Figure 11. RA cases with other diseases.

RA cases having poor health. These cases can be considered as severe cases of rheumatoid arthritis disease and various factors can be involved. Around 14 (35%) of the cases were of fair overall health and only 10 (25%) were of good health. No patient included in this study had excellent health. Extra-articular manifestations other than anemia have been reported in about 15 to 25% of individuals with rheumatoid arthritis (Turesson et al., 2003). Al-Ghamdi and Attar (2009) conducted a research to analyze the frequency and influence of other complicated diseases in RA population. According to this report, anemia is very consistently reported to be present (31%) in rheumatoid patients at RA care centers. In this present study, results are in accordance to Al-Ghamdi's work (Figure 11). One (2.5%) RA case was found to have asthma, three (7.5%) had diabetes, five (12.5%) had heart diseases, nine (22.5%) had blood pressure and 15 (37.5%) of the RA cases had anemia.

Radiological assessments are periodically done to the rheumatoid arthritis patients to observe the prevailing deformity and degeneracy of joint bones (Keelgren and Lawrence, 1957). X-ray radiographs were observed during clinical analysis from each RA case. These radiographs showed various degrees of deformity of joints. Age is an important factor and it is investigated to study the course of the rheumatoid arthritis disease. In

this study, age groups 31 to 35 and 36 to 40 both accounted 30% of total RA cases. Boey et al. (1987) reported similar results while studying patterns of disease in RA patients and found that age group 31 to 40 (27%) had most RA patients. Tobacco consumption is considered as a serious risk factor for many diseases including rheumatoid arthritis (Stolt et al., 2003). Although some recent studies show a high number of smokers in rheumatoid arthritis population, there was no smoker identified in this study. Several reasons can be accounted, such as there was much larger ratio of females in rheumatoid arthritis cases and comparatively very small ratio of males. Culturally in this part of the world, women do not smoke, and there was no smoker among male RA cases. Rheumatoid factor was found to be strongly associated with rheumatoid disease, although four (10%) out of 40 clinically diagnosed RA cases were found to be negative for RF test though they fulfilled the ACR criteria for rheumatoid arthritis diagnosis. There can be several factors involved. One of them might be the prolonged medication that accounted for RF seronegative rheumatoid arthritis patients.

It is very important to continue identification of new diagnostic elements such as autoantibodies, antigens and other immunogenic elements involved in the etiology of the disease. This provides new insight into pathogenesis of the disease and provides a better understanding of the disease process to ensure the future possibilities for the development of new therapeutic strategies to help RA patients to gain the quality of life. There are other autoimmunogenic elements having specificity with rheumatoid arthritis, such as anti CCP, anti-RA33, anti collagen and anti GP1 antibodies (Steiner and Smolen, 2002). Deegan (1980) suggested that anti nDNA antibodies were diagnostically important. The antinuclear antibodies for double stranded DNA were found to be present in some RA cases but there was no significant specificity found between nDNA antibodies for RA cases (Carolyn and Norman, 1979). Although it is considered to be a routine laboratory test for SLE for years, the association of nDNA antibodies and rheumatoid arthritis was estimated through indirect immunoflouresence assay (Juby et al., 1994). There were five (13.8%) positive rheumatoid arthritis cases in this study. Some studies showed the possibility that the antinuclear antibodies maybe present in both SLE and RA patients (22). Although there was no significant association found between nDNA antibodies and rheumatoid arthritis disease, indeed it provides an addition to scientific knowledge in the fields of immunology and rheumatology.

Conflict of Interests

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

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