IJOEAR

International Journal of Environmental & Agriculture Research

www.ijoear.com

ISSN
2454-1850

Volume-3, Issue-10, October 2017

Preface

We would like to present, with great pleasure, the inaugural volume-3, Issue-10, October 2017, of a scholarly journal, *International Journal of Environmental & Agriculture Research*. This journal is part of the AD Publications series *in the field of Environmental & Agriculture Research Development*, and is devoted to the gamut of Environmental & Agriculture issues, from theoretical aspects to application-dependent studies and the validation of emerging technologies.

This journal was envisioned and founded to represent the growing needs of Environmental & Agriculture as an emerging and increasingly vital field, now widely recognized as an integral part of scientific and technical investigations. Its mission is to become a voice of the Environmental & Agriculture community, addressing researchers and practitioners in below areas

Environmental Research:

Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment, Environment society, Agricultural engineering, Animal science, Agronomy, including plant science, theoretical production ecology, horticulture, plant, breeding, plant fertilization, soil science and all field related to Environmental Research.

Agriculture Research:

Agriculture, Biological engineering, including genetic engineering, microbiology, Environmental impacts of agriculture, forestry, Food science, Husbandry, Irrigation and water management, Land use, Waste management and all fields related to Agriculture.

Each article in this issue provides an example of a concrete industrial application or a case study of the presented methodology to amplify the impact of the contribution. We are very thankful to everybody within that community who supported the idea of creating a new Research with *IJOEAR*. We are certain that this issue will be followed by many others, reporting new developments in the Environment and Agriculture Research Science field. This issue would not have been possible without the great support of the Reviewer, Editorial Board members and also with our Advisory Board Members, and we would like to express our sincere thanks to all of them. We would also like to express our gratitude to the editorial staff of AD Publications, who supported us at every stage of the project. It is our hope that this fine collection of articles will be a valuable resource for *IJOEAR* readers and will stimulate further research into the vibrant area of Environmental & Agriculture Research.

Mukesh Arora
(Editor-in Chief)

Dr. Bhagawan Bharali
(Managing Editor)

Fields of Interests

Agricultural Sciences	
Soil Science	Plant Science
Animal Science	Agricultural Economics
Agricultural Chemistry	Basic biology concepts
Sustainable Natural Resource Utilisation	Management of the Environment
Agricultural Management Practices	Agricultural Technology
Natural Resources	Basic Horticulture
Food System	Irrigation and water management
Crop Production	
Cereals or Basic Grains: Oats, Wheat, Barley, Rye, Triticale, Corn, Sorghum, Millet, Quinoa and Amaranth	Oilseeds: Canola, Rapeseed, Flax, Sunflowers, Corn and Hempseed
Pulse Crops: Peas (all types), field beans, faba beans, lentils, soybeans, peanuts and chickpeas.	Hay and Silage (Forage crop) Production
Vegetable crops or Olericulture: Crops utilized fresh or whole (wholefood crop, no or limited processing, i.e., fresh cut salad); (Lettuce, Cabbage, Carrots, Potatoes, Tomatoes, Herbs, etc.)	Tree Fruit crops: apples, oranges, stone fruit (i.e., peaches, plums, cherries)
Tree Nut crops: Hazlenuts. walnuts, almonds, cashews, pecans	Berry crops: strawberries, blueberries, raspberries
Sugar crops: sugarcane. sugar beets, sorghum	Potatoes varieties and production.
Livestock Production	
Animal husbandry	Ranch
Camel	Yak
Pigs	Sheep
Goats	Poultry
Bees	Dogs
Exotic species	Chicken Growth
Aquaculture	
Fish farm	Shrimp farm
Freshwater prawn farm	Integrated Multi-Trophic Aquaculture
Milk Production (Dairy)	
Dairy goat	Dairy cow
Dairy Sheep	Water Buffalo
Moose milk	Dairy product
Forest Products and Forest management	
Forestry/Silviculture	Agroforestry
Silvopasture	Christmas tree cultivation
Maple syrup	Forestry Growth
Mechanical	
General Farm Machinery	Tillage equipment
Harvesting equipment	Processing equipment
Hay & Silage/Forage equipment	Milking equipment
Hand tools & activities	Stock handling & control equipment
Agricultural buildings	Storage

Agricultural Input Products	
Crop Protection Chemicals	Feed supplements
Chemical based (inorganic) fertilizers	Organic fertilizers
Environmental Science	
Environmental science and regulation	Ecotoxicology
Environmental health issues	Atmosphere and climate
Terrestric ecosystems	Aquatic ecosystems
Energy and environment	Marine research
Biodiversity	Pharmaceuticals in the environment
Genetically modified organisms	Biotechnology
Risk assessment	Environment society
Theoretical production ecology	horticulture
Breeding	plant fertilization

Board Members

Dr. V K Joshi

Professor V.K.Joshi is M.Sc., Ph.D. (Microbiology) from Punjab Agricultural University, Ludhiana and Guru Nanak Dev University, Amritsar, respectively with more than 35 years experience in Fruit Fermentation Technology, Indigenous fermented foods, patulin ,biocolour ,Quality Control and Waste Utilization. Presently, heading the dept. of Food Science and Technology in University of Horticulture and Forestry, Nauni-Solan (HP), India.

Mr. Aklilu Bajigo Madalcho

Working at Jigjiga University, Ethiopia, as lecturer and researcher at the College of Dry land Agriculture, department of Natural Resources Management.

Dr. Vijay A. Patil

Working as Assistant Research Scientist in Main Rice Research Centre, Navsari Agricultural University, Navsari. Gujarat- 396 450 (India).

Dr. S. K. Jain

Presently working as Officer Incharge of All India Coordinated Sorghum Improvement Project, S. D. Agricultural University, Deesa, Gujarat.

Dr. Salvinder Singh

Presently working as Associate Professor in the Department of Agricultural Biotechnology in Assam Agricultural University, Jorhat, Assam.

Dr. Salvinder received MacKnight Foundation Fellowship for pre-doc training at WSU, USA – January 2000- March 2002 and DBT oversease Associateship for Post-Doc at WSU, USA – April, 2012 to October, 2012.

Mr. Anil Kumar

Working as Junior Research Officer/Asstt. Prof. in the dept. of Food Science & Technology in Agriculture & Technology, Pantnagar.

Table of Contents

Detection of *Parapoxvirus* in goats during contagious ecthyma outbreak in Ceará State, Brazil by transmission electron microscopy techniques

Marcia Helena Braga Catroxo[1*], Martins A.M.C.R.P.F.[2], Souza F.[3]

Electron Microscopy Laboratory, Research and Development Center in Animal Health, Biological Institute, São Paulo, SP, Brazil

*Corresponding Author: Marcia Helena Braga Catroxo
Biological Institute of São Paulo, SP, Brazil

***Abstract*—** *Contagious ecthyma or contagious pustular dermatitis, is a viral skin disease that occurs in sheep, goats and wild ruminants and is characterized by the formation of papules, nodules or vesicles that progress into thick crusts or heavy scabs on the lips, gingiva and tongue, caused by a member of the Parapoxvirus genus. Humans are occasionally affected constituting important zoonosis. The disease not only has an economic impact on farmers worldwide but also has a considerable negative effect on animal welfare. In this study, a contagious ecthyma outbreak which occurred in one flock with 90 goats located in the Ceará State, Brazil, was described. Twenty-two goats older than 6 months were affected. The animals presented crusted lesions on the buccal region, tongue, udder and teats, which began with swelling in the mouth area. Dried crusts and serum collected were processed for transmission electron microscopy utilizing, negative staining (rapid preparation), Immunocytochemistry (immunolabelling with colloidal gold particles) and resin embedding techniques. At the Philips EM 208 transmission electron microscopy all the samples were analyzed by negative staining technique and a great number of parapoxvirus particles ovoid or cylindrical, showing two morphological forms, a mulberry (M) form with a distinctive crisscross filament pattern derived from the superimposition of upper and lower virion surfaces and a capsular (C) form caused by stain penetration and distention of the virion core, measuring 300 x 180 nm was observed. Antigen antibody reaction was increased by the colloidal gold particles. In the ultrathin sections of crusts, we verified the presence of three types of intracytoplasmic inclusion bodies, type A or Bollinger inclusion bodies, outlined by membrane, presented in it is interior, oval, mature or complete viral particles, measuring on the average, 225nm x 130 nm, showing an inner dumbell-shaped core, two lateral bodies and an external envelope, or cigar shaped core. In the type B electron dense inclusions bodies, were visualized parapoxvirus particles budding of dense and amorphous material. Fibrillar intracytoplasmic inclusions were also found located between the virions, consisting of groups of fibrils, arranged in groups or concentrically in the middle of the granular material. Intracytoplasmic vesicles outlined by membranes, measuring 560 x 420 nm, containing granular material in its interior were also observed. The nuclei showed an aspect deformed.*

Keywords*— *Parapoxvirus, Goats, Transmission electron microscopy.

I. INTRODUCTION

Genus *Parapoxvirus* has four members, *Orf virus* (ORFV), *Bovine papular stomatitis virus* (BPSV), *Pseudocowpoxvirus* (PCPV) and *Parapoxvirus of red deer in New Zealand* (PVNZ). The ORFV is the prototype member.

Enveloped virions present with an ovoid shape and the spiral tubule surrounding the virion surface can be clearly distinguished by electron microscopy from orthopoxviruses (OPV) because of their regular surface structure. The particles of ORF virus are 260 nm length by 160 nm wide (Damon, 2007; Moss, 2007).

The virus genome includes linear double-stranded DNA about 138 kb length with 64% G+C content, which contains 132 putative genes that included 89 highly conserved genes and some variable genes. The envelope gene (P2L) of the virus encodes a highly immunogenic major envelope protein widely used for molecular characterization and phylogenetic analysis of strains of the virus (Inoshima et al., 2000; Delhon et al., 2004; Mercer et al., 2006; Zhang et al., 2010).

ORF virus is the etiological agent of the contagious ecthyma or contagious pustular dermatitis, a viral skin disease that occurs in sheep, goats and wild ruminants, characterized by the formation of papules, nodules or vesicles that progress into thick crusts or heavy scabs on the lips, gingiva, tongue, eyelids and feet and occasionally on the teats (Vikoren et al., 2008).

Susceptible animals usually develop the first signs of the disease 4 to 7 days after exposure that persists for 1 to 2 weeks or

for large periods (Haig & Mercer, 1998).

The mortality occurs, especially in young sucking lambs, due to an incidence of dehydration and starvation, as the pain and distortion of the lips and mouth preclude the lamb from sucking (Mombeni et al., 2012).

The mortality rate is usually low but it can reach 93% in lambs with secondary bacterial or fungal infections (Haig & Mercer, 1998; Zhao et al., 2010).

The infection is spread by direct and indirect contact from infected animals or by contact with infected tissue or saliva containing the virus (Lojkic et al., 2007).

Outbreaks occur more frequently during periods of extreme temperatures such as late summer and winter (Robinson & Balassu, 1981; Gokce et al., 2005).

Some infected animals become carriers and shed the virus for a long period (Mombeni et al., 2012).

Humans are occasionally affected constituting important zoonosis (Al Salam et al., 2008; Kitchen et al., 2013; Turan et al., 2013).

The disease not only has an economic impact on farmers worldwide but also has a considerable negative effect on animal welfare. Infected animals are sickly, fail to thrive, and are more susceptible to bacterial infections (Gallina et al., 2008).

Outbreaks of the disease have been reported in several countries, such as China (Zhang et al., 2010; Li et al., 2012), Croatia (Lojkic et al., 2010), Italy (Gallina et al., 2008), Japan (Inoshima et al, 2001), Taiwan (Chen et al., 2007) and Greece (Billinis et al., 2012).

In Brazil, a few reports were described in the states of Mato Grosso, MT (Abrahão et al., 2009); Pará, PA (Oliveira et al., 2012); Paraíba, PB (Nóbrega et al., 2008); Minas Gerais, MG, Rio Grande do Sul, RS e Pernambuco, PE (Mazur et al., 2000).

Considering the efficiency and speed of transmission electron microscopy techniques, this study aimed to detect the presence of parapoxvirus in skin lesions of goats during outbreak of contagious ecthyma first occurred in Ceará state, Brazil.

II. MATERIAL AND METHOD

2.1 Description of the outbreak

In this study, a contagious ecthyma outbreak occurred in the year 2011 in a flock with 90 goats located in the Ceará State, Brazil. Twenty-two goats older than 6 months were affected. The animals presented crusted lesions on the buccal region, tongue, udder and teats, which began with swelling in the mouth area that progressed to papules, vesicles, pustules and crusts. The mortality rate was 0% and the animals recovered 30 days after the onset of symptoms.

2.2 Transmission Electron Microscopy

Fragments of skin lesions and dry crusts and serum collected were processed for transmission electron microscopy utilizing negative staining (rapid preparation), immunocytochemistry (immunolabeling with colloidal gold particles) and resin embedding techniques.

2.2.1 Negative staining technique (rapid preparation)

In the negative staining process, the fragments of skin lesions and dry crusts were suspended in phosphate buffer 0.1 M and pH 7.0 and placed in contact with metallic grids. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

2.2.2 Immunocytochemistry technique.

At the immunolabeling technique with colloidal gold particles for negative staining, the copper grids were placed in contact with viral suspension of the samples of skin lesions and dry crusts and, after removing the excess with filter paper, the same were put on specific primary antibody drops. After further washing in PBS drops, the grids were incubated in protein A drops, in association with 10 nm colloidal gold particles (secondary antibody). Grids were then contrasted with 2% ammonium molybdate at pH 5.0 (Knutton, 1995).

2.2.3 Resin embedding technique.

Fragments of dry crusts were fixed in 2.5% glutaraldehyde in 0.1 M, pH7.0 phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in cetonic series, the fragments were embedded in Spurr resin (González-Santander 1969; Luft, 1961). Ultrathin sections were cut on the LKB ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson, 1958; Reynolds, 1963).

All grids submitted to the above reactions were observed in a Philips EM 208 electron microscope, at 80 kV.

III. RESULTS AND DISCUSSION

3.1 Transmission Electron Microscopy

3.1.1 Negative Staining Technique (Rapid Preparation)

At the Philips EM 208 transmission electron microscope all the skin lesions and dry crusts samples examined by the negative staining technique showed a large number of parapoxvirus particles, ovoid or cylindrical, measuring 300 nm of length x 180 nm in diameter. Two morphological forms were visualized, a mulberry (M) form (fig. 1, big arrow) with a distinctive crisscross filament pattern derived from the superimposition of upper and lower virion surfaces and a capsular (C) form (fig. 2, minor arrow) caused by stain penetration and distention of the virion core.

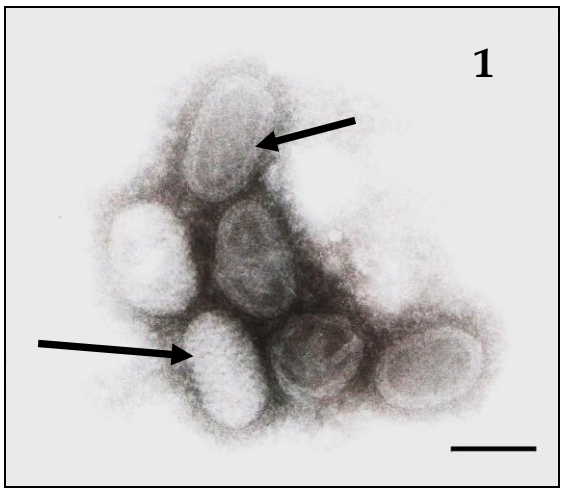

FIGURE 1: ELECTRON MICROGRAPH OF PARAPOXVIRUS IN CRUSTS SUSPENSION BY NEGATIVE STAINING, SHOWING A MULBERRY (M) FORM (BIG ARROW) WITH A DISTINCTIVE CRISSCROSS FILAMENT PATTERN DERIVED FROM THE SUPERIMPOSITION OF UPPER AND LOWER VIRION SURFACES AND A CAPSULAR (C) FORM (MINOR ARROW) CAUSED BY STAIN PENETRATION AND DISTENTION OF THE VIRION CORE. BAR: 180 nm.

3.1.2 Immunocytochemistry Technique.

In the immunocytochemistry technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the parapoxvirus in all samples of skin lesions and dry crusts fragments (Figure 2, arrow), confirming the results of negative staining technique.

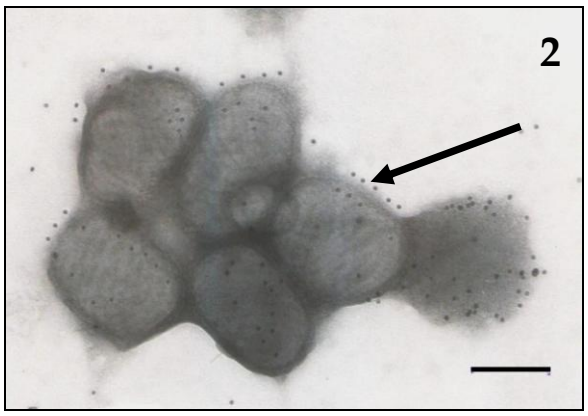

FIGURE 2: ELECTRON MICROGRAPH OF PARAPOXVIRUS GROUPING ENHANCED BY COLLOIDAL GOLD PARTICLES IN THE IMMUNOCYTOCHEMISTRY TECHNIQUE (ARROW). BAR: 140 nm.

3.1.3 Resin Embedding Technique.

In the ultrathin sections of crusts, we verified the presence of three types of intracytoplasmic inclusion bodies, type A or Bollinger inclusion bodies, type B or amorphous inclusion bodies and fibrillar inclusion bodies. Type A or Bollinger inclusion bodies **(figs. 3,4)**, outlined by membrane, presented in it is interior, oval, mature or complete viral particles **(figs. 3,4,5, arrow)**, measuring on the average, 225 nm of lenght x 130 nm of diameter, showing an inner dumbell-shaped core **(fig.5, big arrow)**, two lateral bodies **(fig. 5, minor arrow)** and an external envelope **(fig. 5, (arrowhead)**, or more often cigar shaped core **(fig. 5, arrow blue)**. In the type B electron dense inclusions bodies, were visualized parapoxvirus particles budding of dense and amorphous material **(figs. 6,7, arrow)**. Fibrillar intracytoplasmic inclusions were also found located between the virions, consisting of groups of fibrils, arranged in groups **(fig. 8, big arrow)** or concentrically in the middle of the granular material **(fig. 6, blue arrow)**, with possible association with the initial stage of formation of the virion **(fig. 8, minor arrow)**. These fibrils measured around 7-10 nm in diameter. Intracytoplasmic vesicles outlined by membranes, measuring 560 x 420 nm of diameter, containing granular material in its interior were also observed **(fig. 9, arrow)**. The nuclei showed a deformed aspect **(fig. 10)**.

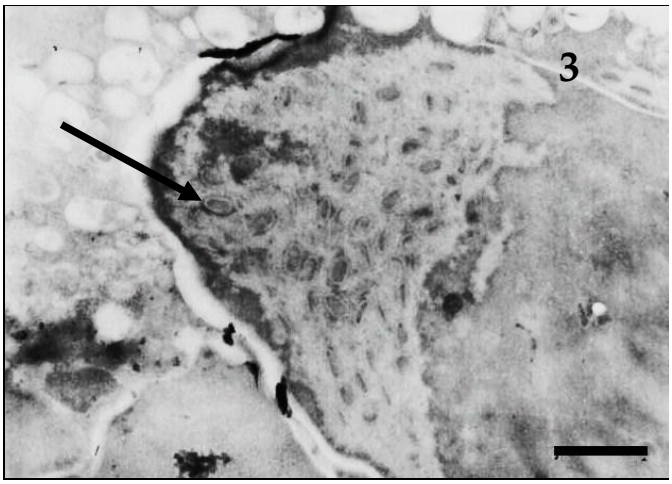

FIGURE 3: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. OBSERVE TYPE A OR BOLLINGER INTRACYTOPLASMIC INCLUSION BODIES, PRESENTED IN ITS INTERIOR MATURE PARTICLES (ARROW). BAR: 640 nm.

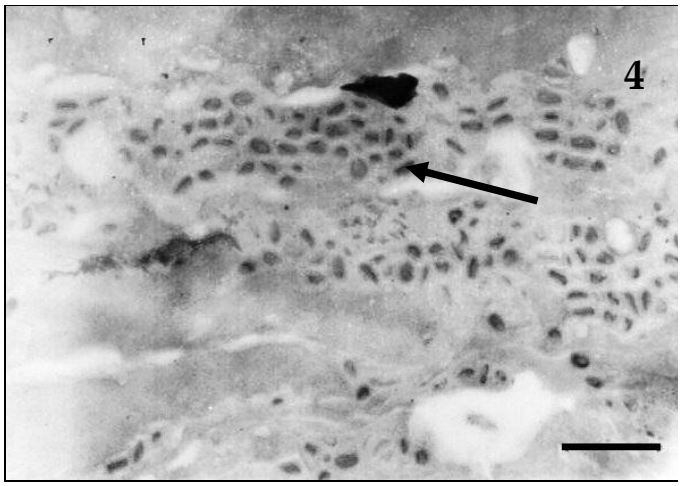

FIGURE 4: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. TYPE A OR BOLLINGER INCLUSION BODIES CONTAINING GREAT NUMBER OF PARAPOXVIRUS PARTICLES (ARROW). BAR: 720 nm.

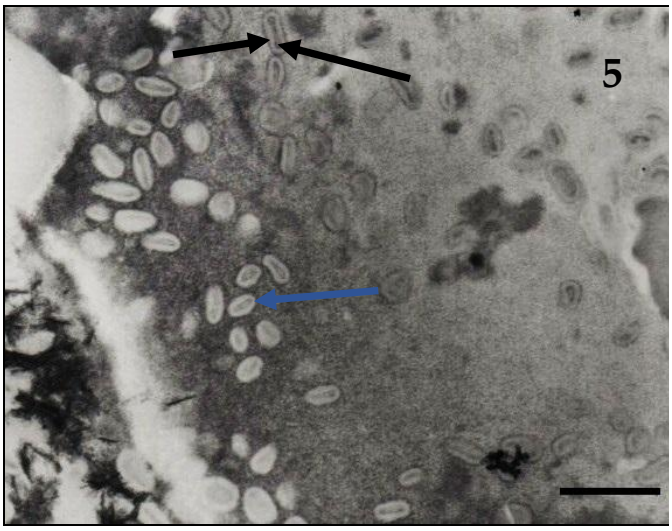

FIGURE 5: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. COMPLETE PARAPOXVIRUS PARTICLES SHOWING AN INNER DUMBELL-SHAPED CORE (BIG ARROW), TWO LATERAL BODIES (MINOR ARROW) AND AN EXTERNAL ENVELOPE (ARROW HEAD), OR CIGAR SHAPED CORE (ARROW BLUE). BAR: 540 nm.

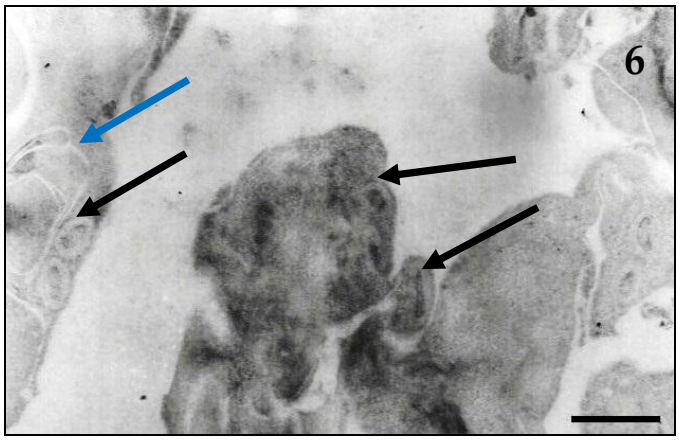

FIGURE 6: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. TYPE B INCLUSION BODIES SHOWING PARAPOXVIRUS BUDDING OF DENSE AND AMORPHOUS MATERIAL (ARROW) AND FIBRILLAR INCLUSIONS (BLUE ARROW). BAR: 280 nm.

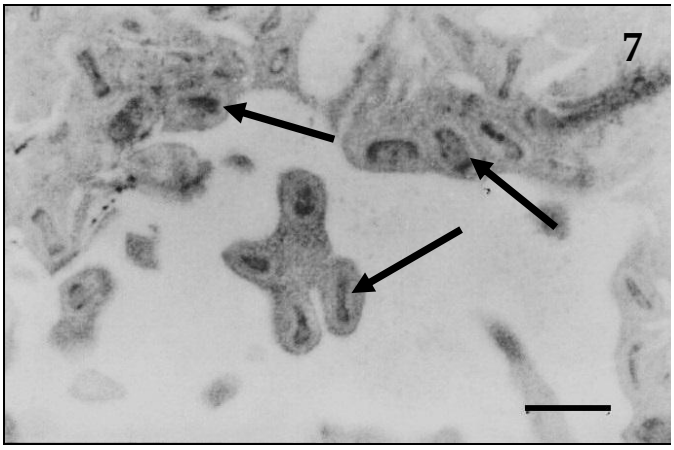

FIGURE 7: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. TYPE B INCLUSION BODIES. OBSERVE GREAT NUMBER OF PARAPOXVIRUS PARTICLES BUDDING OF AMORPHOUS MATERIAL (ARROW). BAR: 260 nm

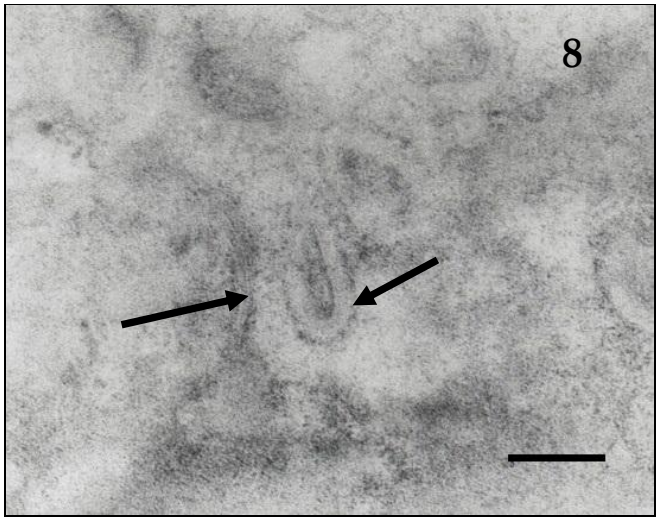

FIGURE 8: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. FIBRILLAR INCLUSION CONSTITUTED BY GROUPS OF FIBRILS (BIG ARROW), WITH POSSIBLE ASSOCIATION WITH THE INITIAL STAGE OF FORMATION OF THE VIRION (MINOR ARROW). BAR: 140 nm

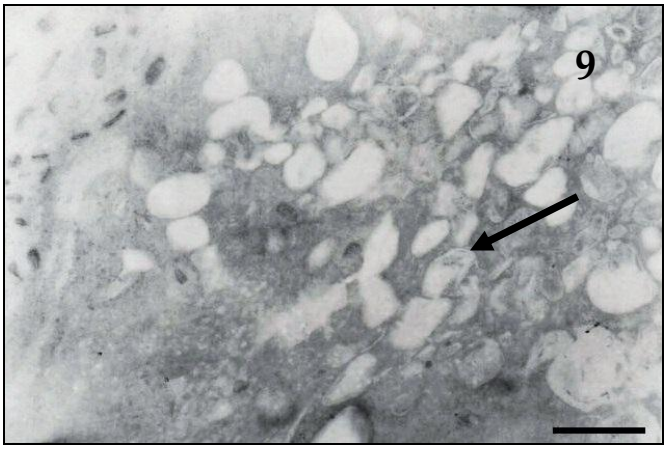

FIGURE 9: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. VESICLES CONTAINING GRANULAR MATERIAL IN ITS INTERIOR (ARROW). BAR: 560 nm.

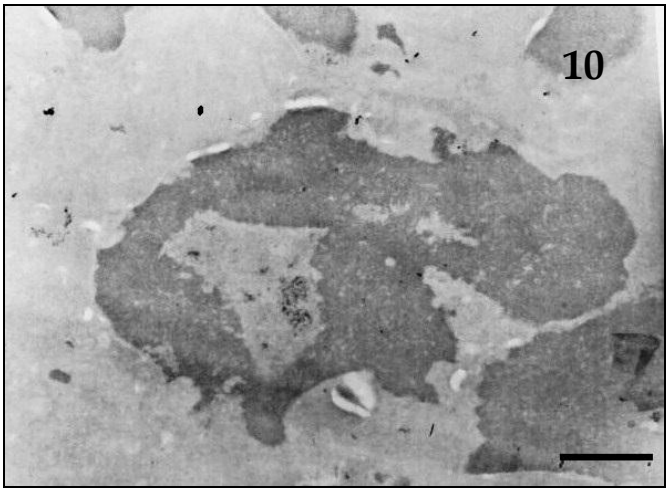

FIGURE 10: ULTRATHIN SECTION OF THE CRUSTS FRAGMENTS. OBSERVE NUCLEI WITH DEFORMED ASPECT. BAR: 720 nm.

In the study, a contagious ecthyma outbreak, which occurred in one flock with 90 goats located in the Ceará State, was described.

The clinical signs were characterized by lesions on the buccal region, tongue, udder and teats, with swelling in the mouth area that progressed to papules, vesicles, pustules and crusts.

Similar clinical signs were reported by other authors in research on contagious ecthyma in goats and sheeps (Inoshima et al., 2001; Nitsche et al., 2006; Chan et al., 2007; OIE, 2007; Abrahão et al., 2009; Oem et al., 2009; Lojkic et al., 2010; Zhang et al., 2010; Zhao et al.,2010; Shao-Peng et al., 2011; Billinis et al., 2012; Li et al., 2012; Mombeni et al., 2012; de Oliveira et al., 2012; Li et al., 2013).

Analogous lesions caused by parapoxvirus were also observed in camels (Gitao, 1994) and in a red deer (Scagliarini et al., 2011). Skin nodules located in head, neck and thorax, were reported in a sea lynx (*Zalophus californianus*) infected with parapoxvirus (Nollens et al., 2006).

The twenty-two affected goats of our study had 6 months of age, in agreement with other studies that showed that the disease affects animals from 3 to 6 months of age (Mombeni et al., 2012; Kinley et al; 2013). Younger animals with 2-3 weeks to 2

months, however, can be seriously affected (Oem et al., 2009; Lojkic et al., 2010). Controversially, Abu & Housawi (2009) and Gallina et al. (2008) stated that in adult goats the clinical signs might be more severe.

The mortality rate was 0% and the animals recovered 30 days after the onset of symptoms.

The low mortality rate is commonly reported, ranging from 0.8% to 24.7% (Chan et al.,2007; Abu & Housawi, 2009; Zhao et al., 2010; Zhang et al., 2010; Shao-Peng et al., 2011; Li et al., 2012; Mombeni et al., 2012). Mortality due to starvation, however, was recorded in 80% of affected lambs owing to suckling difficulty was related (Lojkic et al., 2010). In young, stressed, or immunosuppressed sheep, formation of severe bloody lesions can be fatal (Nitsche et al., 2006).

Of the 90 flock, the disease affected 22 animals (24.4%).

The morbidity rate was also variable among other studies, ranging from 6% to 100% (Chan et al.,2007; Abu & Housawi, 2009; Oem et al., 2009; Lojkic et al., 2010; Zhao et al., 2010; Zhang et al., 2010; Shao-Peng et al., 2011; Li et al., 2012; Mombeni et al., 2012).

The diagnostic was realized by applying the techniques of transmission electron microscopy.

The negative staining technique detected typical parapoxvirus particles, ovoid or cylindrical, measuring 270 nm from length x 180 nm of diameter, morphological description also mentioned in other studies of contagious ecthyma (Gitao, 1994; Mazur et al. 2000; Torfason & Gudnadottir, 2002; Mercer et al., 2006; Nollens et al., 2006; Chan et al., 2007; 2009; Mast & Demestere, 2009; Oem et al., 2009; Cargnelutti et al., 2010; Zhao et al., 2010; Scagliarini et al., 2011; Shao-Peng et al., 2011; Kitchen et al., 2013). The two morphological forms, the mulberry (M) form, non-enveloped, with distinctive crisscross filament pattern and a capsular (C) form, enveloped, which we have visualized, were also described by Kitchen et al. (2013).

In ultrathin sections of crusts, we verified the presence of type A or Bollinger intracytoplasmic inclusion bodies, containing oval, mature or complete viral particles, measuring on the average, 225 x 130 nm, showing an inner dumbell-shaped or cigar shaped core, ultrastructural aspects also reported by other authors (Muller et al., 2003; Nollens et al., 2006; Li et al., 2012).

We visualized inclusions of type B or amorphous inclusions, containing immature particles, budding from dense and amorphous material. These type of inclusion were observed in other poxvirus relates (Purcell et al., 1972; Thiele et al, 1979; Sadosiv et al., 1985; Bersano et al., 2003; Catroxo et al., 2009a,b).

In addition, we observed the fibrill intracytoplasmic inclusions previously described in studies on swinepox (Teppema & Boer, 1975; Bersano et al., 2003), avianpox (Catroxo et al., 2009a) and myxomatosis (Catroxo et al., 2009b). These inclusions were located intranuclearly in the case of swinepox (Teppema & Boer, 1975). According to Moss (1996), the initial stage of virion formation occurs in granular and electron-dense areas of the cytoplasm, separated from the cell membrane.

The presence of large numbers of intracytoplasmic vacuoles, surrounded by membranes, containing granular material, as well as deformed nuclei with dense chromatin is in accordance with other researches (Catroxo et al., 2009 a, b).

The immunocytochemistry technique we applied strongly marked the parapoxvirus particles with colloidal gold, allowing the visibility of the antigen-antibody interaction, also used by other researchers to confirm the poxvirus viral strain (Bersano et al., 2003; Catroxo et al., 2009 a,b) and to study the structure and morphogenesis of the ORF virus (Tan et al., 2009).

The diagnosis of contagious ecthyma was effectively concluded through the transmission electron microscopy techniques, being considered a classic tool and of the first line for diagnosis of poxvirus in swabs or crusts where the virus particles with their typical morphology are usually present in sufficient amount (Mercer et al., 2007).

Through this technique, parapoxvirus can easily be differentiated from orthopoxvirus by its different morphologies, with electron microscopy being one of the techniques chosen by the OIE to diagnose contagious ecthyma (Gitao, 1994; OIE, 2007).

In cases of lower viral loads, the problem can be contorned by the application of immuno-specific techniques, such as immunoelectron microscopy or immunolabeling with colloidal gold. Also in cases of not preserved material, the crusts can be processed by the resin embedding technique, followed by ultrathin sections, where in addition to the visualization of the viral particles, the cytopathic effects caused by the virus can be observed on the host cells (Catroxo et al., 2009 a,b).

Considering that contagious ecthyma is a zoonosis, with public health implication, viral detection becomes important both in animals and in humans. Detection of parapoxviruses is fundamental for the exclusion of other rash-causing illnesses, in areas both veterinary and medical (Nitsche et al, 2006).

Contagious ecthyma may predispose animals to bacterial mastitis and it is therefore important to treat the lesions on the teats (OIE, 2008).

The introduction of new ORFV strains in Brazil seems to occur through the import of animals in order to improve herd genetics (Abrahão et al., 2009).

In order to avoid the risk of disease entry into herds and prevent economic losses resulting from it, important measures should be taken in livestock breeding. Proceed annual vaccination of young animals when the disease is endemic, always apply quarantine in the new animals before introducing them to the rest of the herd, surveillance on the introduction of diseased animals, and avoid stress factors and coarse grazing that cause abrasions on the skin and mucous membranes of the animals, facilitating the entry of the virus (OIE, 2007).

IV. CONCLUSION

Considering that contagious ecthyma is a highly contagious, zoonotic, viral skin disease that affects sheep and goats, causing fail to thrive and decreases in production, the application of transmission electron microscopy techniques in routine or during outbreaks of the disease may help to develop measures for prevention and control of contagious ecthyma, collaborating with the National Goat and Sheep Health Program.

REFERENCES

[1] Abrahão J.S., Campos R.K., Trindade G.S., Guedes M.I., Lobato Z.I., Mazur C., Ferreira P.C., Bonjardim C.A. and Kroon E.G. 2009. Detection and phylogenetic analysis of Orf virus from sheep in Brazil: a case report. Virol. J. 6:47:1-4.

[2] Abu E.M. and Housawi F.M. 2009. Drastic cutaneous multi-focal orf infection in goats, causing severe dysfunctioning. Rev. Sci. Tech. 28:1025-1029.

[3] Al-Salam S., Nowotny N., Sohail M.R., Kolodziejek J., and Berger T.G. 2008. Ecthyma contagiosum (orf) – report of a human case from the United Arab Emirates and review of the literature. J. Cutan. Pathol. 35: 603–607.

[4] Bersano J. G., Catroxo M. H. B., Villalobos E. M. C., Leme M. C. M., Martins A. M. C. P. F., Peixoto Z. M. P.; Portugal M. A. S. C., Monteiro R. M., Ogata R. A. and Curi N. A. 2003. Varíola suína: estudo sobre a ocorrência de surtos nos estados de São Paulo e Tocantins, Brasil. Arq. Inst. Biol. 70:269-278.

[5] Billinis C., Mavrogianni V.S., Spyrou V. and Fthenakis G.C. 2012. Phylogenetic analysis of strains of Orf virus isolated from two outbreaks of the disease in sheep in Greece. Virol. J. 9:24:1-8.

[6] Brenner S. and Horne R. W. 1959. A negative staining method for high resolution electron microscopy of viruses. Biochem. Biophys. Acta. 34:103-110.

[7] Cargnelutti J.P., Schmidt C., Weiblen R. and Flores E.F. 2010. Ectima contagioso. Boletim Informativo do Deparatmento de Medicina Veterinária do Centro de Ciências Rurais da Universidade Federal de Santa Maria (UFSM), RS, 4:1-4.

[8] Catroxo M.H.B., Pongiluppi T., Melo N. A., Milanelo L., Petrella S., Martins A.M.C.P.F. and Rebouças M.M. 2009a. Identification of poxvirus under transmission electron microscopy during outbreak period in wild birds, in São Paulo, Brazil. Int. J. Morphol. 27:577-585.

[9] Catroxo M.H.B., Bersano J. G., Martins A. M. C. P. F., Petrella S., Portugal M. A. S. C. and Souza O. S. 2009b. Ultrastructural study of poxvirus causing myxomatosis in rabbits, in São Paulo and Santa Catarina, Brazil. Int. J. Morphol. 27:543-552.

[10] Chan, K.-W., Lin J.-W., Lee S.-H., Liao C.-J.,Tsai M.-C., Li Hsu W.-L.,Wong M.-L. and Shih H.-C. 2007. Identification and phylogenetic analysis of orf virus from goats in Taiwan. Virus Genes 35:705–712.

[11] Damon I. *Poxviridae* and their replication. In: Fields Virology, New York, Raven Press Ltd., 2007, pp. 2079-2081.

[12] Delhon G., Tulman, E.R., Afonso C.L., Lu Z., Concha-Bermejillo A., Lehmkuhl H.D., Piccone M.E., Kutish G.F., and Rock D.L. 2004. Genomes of the parapoxviruses Orf virus and bovine papular stomatitis virus. J. Virol. 78:168–177.

[13] Gallina L., Scagliarini L., C. J. McInnes C.J., Guercio A., Purpari G., Prosperi S. and Scagliarini A. 2008. Parapoxvirus in goats: experimental infection and genomic analysis. Vet. Res. Commun. 32: S203–S205.

[14] Gitao C.G. 1994. Outbreaks of contagious ecthyma in camels (*Camelus dromedarius*) in the Turkana district of Kenya. Rev.sci.tech.Off.int.Epiz.13:939-945.

[15] Gokce H.I., Genc O. & Gokce G. 2005. Sero-prevalence of contagious ecthyma in lambs and humans in Kars, Turkey. Turk. J. Vet. Anim. Sci. 29:95-101.

[16] Gonzalez-Santander R. Técnicas de microscopia electrónica en biología. Madrid, Ed. Aguilar, 1969, p.666.

[17] Haig D.M. and Mercer A.A. 1998. Ovine diseases. Orf. Vet. Res. 29:311–326.

[18] Hayat M.A. and Miller S.E. Negative Staining. New York: Mc Graw Hill Publ. Company, 1990, p.235.

[19] Inoshima Y., Morooka A. and Sentsui H. 2000. Detection and diagnosis of parapoxvirus by the polymerase chain reaction. J. Virol. Methods. 84:201-208.

[20] Inoshima Y., Murakami K.,Yokoyama T. and Sentsui H. 2001. Genetic heterogeneity among parapoxviruses isolated from sheep, cattle and Japanese serows (*Capricornis crispus*). J. Gen. Virol. 82:1215–1220.

[21] Kinley G.E., Schmitt C.W. and Stephens-Devall J. 2013. A case of contagious ecthyma (Orf Virus) in a nonmanipulated laboratory dorset sheep (*Ovis aries*). Case Rep. Vet. Med. 1-5.

[22] Kitchen M., Müller H., Zobl A., Windisch A., Romani N., and Huemer H. 2013. Orf virus infection in a hunter in Western Austria, presumably transmitted by game. Acta. Derm. Venereol. 94:212-214.

[23] Knutton S.1995. Electron microscopical methods in adhesion. Methods. Enzymol. 253:145-158.

[24] Li H., Zhu X., Zheng Y., Wang S., Liu Z., Dou Y., Li H., Cai X., and Luo X. 2013. Phylogenetic analysis of two Chinese orf virus isolates based on sequences of B2L and VIR genes. Arch. Virol.158:1477–1485.

[25] Li W., Ning Z., Hao W., Song D., Gao F., Zhao K., Liao X., Li M., Rock D.L. and Luo S. 2012. Isolation and phylogenetic analysis of orf virus from the sheep herd outbreak in northeast China. BMC Vet. Res. 8:229:1-13.

[26] Lojkic I., Zeljko Cac Z., Beck A., Bedekovic T., Cvetnic Z. and Sostari B. 2010. Phylogenetic analysis of Croatian orf viruses isolated from sheep and goats. Virol. J. 7:314, 2-7.

[27] Luft J.H. 1961. Improvements in an epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414.

[28] Madeley C.R. 1997. Origins of electron microscopy and virus diagnosis. J. Clin. Pathol. 50:454-456.

[29] Mast J. and Demeestere L. 2009. Electron tomography of negatively stained complex viruses: application in their diagnosis. Diag. Pathol. 4:5-7.

[30] Mazur C., Ferreira I.I., Rangel Filho F.B. and Galle R. 2000. Molecular characterization of Brazilian isolates of orf virus. Vet. Microbiol. 73:253-259.

[31] Mercer A.A., Schmidt A. and Weber O. Poxviruses. In: Birkhauser Advances in Infectious Diseases, Birkhauser Verlag, Basel, 2007, pp. 1–45, pp. 113–166, pp. 355–373.

[32] Mercer A.A., Ueda N., Friederichs S., Hofmann K., Fraser K.M., Bateman T. and Fleming S.B. 2006. Comparative analysis of genome sequences of three isolates of Orf virus reveals unexpected sequence variation. Virus Res.116:146-158.

[33] Mombeni E.G., Mousavi M.B., Ranjbaran I., Chanani Z., Hoseini M., Davoudi A., Rahnama A., Sedeh N.S. and Mombeini M.G. 2012. Prevention and treatment of contagious ecthyma in sheep and goat by goat-pox vaccine in Khuzestan Province, Iran. Bull. Env. Pharmacol. Life Sci. 1:69- 72.

[34] Moss B. *Poxviridade*: the viruses and their replication, in: Fields Virology, v.2, Fields B.N, Knipe D.M., Howley P.M. Eds. Philadelphia: Lippincott-Raven, 1996, pp.2637-2671.

[35] Muller G., Groters S., Siebert U., Rosenberger T., Driver J., Konig M., Becher P., Hetzel U. and Baumgartner W. 2003. Parapoxvirus infection in harbor seals (*Phoca vitulina*) from the German North Sea. Vet. Pathol.40:445–454.

[36] Nitsche, A., Buttner M., Wilhelm S., Pauli G. and Meyer H. 2006. Real-Time PCR detection of parapoxvirus DNA. Clin. Chem. 52:316-319.

[37] Nóbrega Jr J.E., Macedo J.T.S.A., Araújo J.A.S., Dantas A.F.M., Soares M.P. and Riet-Correa F. 2008. Ectima contagioso em ovinos e caprinos no semi-árido da Paraíba. Pesq. Vet. Bras. 28:135-139.

[38] Nollens H.H., Jacobson E.R., Gulland F.M.D., Beusse D.O., Bossart G.D., Hernandez J.A., Klein P.A. and Condit R.C. 2006. Pathology and preliminary characterization of a parapoxvirus isolated from a California sea lion (*Zalophus californianus*). J. Wildl. Dis. 42:23–32.

[39] Oem J.-K., Roh I.-S, Lee K.-H., Lee K.-K., Kim H.-R., Jean Y.-H., and Lee O.-S. 2009. Phylogenetic analysis and characterization of Korean orf virus from dairy goats: case report. Virol. J. 6:167:1-5.

[40] OIE Contagious Ecthyma. Manual of diagnostic for terrestrial animals. The Center for Food Security & Public Health. OIE, Paris, France, 2007. P.1-4.

[41] Oliveira C.H.S., Assis F.L., Neto J.D.B., Oliveira C.M.C., Lopes C.T.A., Bomjardim H.A., Vinhote W.M.S., Silva A.G.M., Abrahão J.A. and Kroon E.G. 2012. Multifocal cutaneous orf virus infection in goats in the Amazon region, Brazil. Vector Borne Zoonotic Dis. 12:336-340.

[42] Purcell D.A., Clarke J.K. McFerran B.J. and Hughes D.A. 1972. The morphogenesis of pigeonpox virus. J. Gen. Virol.15:79-83.

[43] Reynolds E.S. 1963.The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17:208-212.

[44] Robinson A.J. and Balassu T.C.1981. Contagious pustular dermatitis (ORF). Vet. Bull. 52:771-782.

[45] Sadosiv E.C. Chang P. W. and Gulka G. 1985. Morphogenesis of canary poxvirus and its entrance into inclusion bodies. Am.J.Vet.Res. 46:529-535.

[46] Scagliarini A., Vaccari F., Turrini F., Bianchi A., Cordioli P. and Lavazza A. 2011. Parapoxvirus infections of red deer, Italy. Emerg. Inf. Dis. 17:684-687.

[47] Shao-peng G.U., Xin-tao S., Zhong-yong S., Zhong-bing W. and Ming-xue Z. 2011. Identification and phylogenetic analysis of an orf virus isolated from an outbreak in boer goat in Shanxi Province. Agr.Sci. China. 10:946-953.

[48] Tan J.L., Ueda N., Mercer A.A. and Fleming S.B. 2009. Investigation of ORF virus structure and morphogenesis using recombinants expressing FLAG-tagged envelope structural proteins: evidence for wrapped virus particles and egress from infected cells. J.Gen.Virol. 90:614-625.

[49] Teppema J.S. and de Boer G.F. 1975. Ultrastructural aspects of experimental swinepox with special reference to inclusion bodies. Arch. Virol. 49:151-163.

[50] Thiele J. Kiel H. and Adolphs H.D. 1979. Avian pox virus. An ultrastructural study on a cherrug falcon. Brief report. Arch. Virol. 62:77-82.

[51] Torfason E.G. and Gudnadottir S. 2002. Polymerase chain reaction for laboratory diagnosis of Orf virus infections. J. Clin. Virol. 24:79–84.

[52] Turan E., Yesilova Y. and Ucmak D. 2013. A case of orf (ecthyma contagiosum) with multiple lesions. J. Pak. Med. Assoc. 63:786-787.

[53] Vikoren T., Lillehaug A., Akerstedt J., Bretten T., Haugum M. and Tryland M. 2008. A severe outbreak of contagious ecthyma (orf) in a free-ranging musk ox (*Ovibos moschatus*) population in Norway. Vet. Microbiol.127:10-20.

[54] Watson M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophyis. Biochem. Cytol. 4:475-478.

[55] Zhang K., Shang Y., Jin Y., Wang G., Zheng H., He J., Lu Z. and Liu X. 2010. Diagnosis and phylogenetic analysis of Orf virus from goats in China: a case report. Virol. J. 7:121.

[56] Zhao K., Song D., He W., Lu H., Zhang B., Li C., Chen K. and Gao F. 2010. Identification and phylogenetic analysis of an Orf virus isolated from an outbreak in sheep in the Jilin province of China. Vet. Microbiol. 142:408-415.

Influence of Plant Density and Mulching on Growth and Yield of Lettuce (*Lactuca sativa* var. *romana* L.)

Milena Yordanova[1], Asen Nikolov[2]

Faculty of Agronomy, University of Forestry, Bulgaria

***Abstract*—** *The aim of the paper was to present the influence of plant density and different types of organic mulch on growth and yield of Romain lettuce. The field experiment was carried out in 2014 in the experimental field of University of Forestry – Sofia, with Romaine lettuce cv. Yellow beauty. The seedlings were planted at a distance of 30 x 30 cm and two different schemes were tested: parallel planting to form a 4-row bed and a chess planting to form a 7-row bed. For the purpose of the study were used different organic mulches, which were waste products from organic agriculture: barley straw (BS), well-rotted horse manure (HM), walnut wood sawdust (WS) and grass windrow (GW) and non-mulched control plots (NMC). The mulching materials were spread manually in a 5-6 cm thick layer, after strengthen the seedlings of Romain lettuce. On the 60th day after planting was the beginning of harvesting. It was found out that the seven-row bed and mulching influenced growth and yield of Romain lettuce. Mulching with HM has a significant effect on growth and yield. In the cultivation of lettuce in combination with horse manure mulch and seven-row bed, the yield increased by 18% compared to the non-mulched control in the same planting scheme and was 2.3 times higher than the non-mulched control in a four-row bed. A significant effect on the mulched variants was established.*

***Keywords*— grass windrow mulch, horse manure mulch, lettuce, seven-row bed, yield.**

I. INTRODUCTION

Lettuce (Lactuca sativa L.) is an annual plant of the Asteraceae family. Due to its short vegetation period and its cold resistance it is one of the first fresh leafy vegetables in the spring. The density of growing plants is one of the factors which affect the yield and quality of produce. Optimum density of plants enabling them to grow uniformly, through effective use of moisture, nutrients, and light, and thus helps to obtain maximum yield. [6]. According to several authors, recommended plant spacing when growing lettuce, usually ranging from 20 to 50 cm between rows and 20 to 45 cm between plants. It has been found that by increasing the spacing of the growing plant height and diameter of the lettuce are greater, leading to an increase in the fresh weight of a plant. At the same time, the reduced number of plants leads to a decrease in the total yield. The significant increase in yield at a high density of plants can only be attributed to the larger number of plants per unit area. [7]. But smaller distances also lead to reduced yields due to competition between plants for light, water, food, etc. [2, 6, 9]

Mulching is an agrotechnical event, which is covering the soil surface with a layer of organic or inorganic materials, which aims to improve the conditions for plant growth. It helps to maintain a constant soil temperature, controls weeds, retain soil moisture and more [7, 11]. The advantage of organic mulch is that they keep a moderate temperature; omit water from precipitation and maintain better soil humidity, [4, 15, 16]; increase total yield; improving soil fertility, as after their plowing at the end of vegetation, they are a valuable source of organic matter [12, 13, 14].

Although the lettuce has a short growing season, mulching favorably affect growth and development. It has been shown that lettuce grown with mulching soil surface increase their height and diameter compared to non-mulching plants. The fresh and dry weight of the plants is increased. The positive effect is probably due to the reduction in the loss of nutrients from washing, improved temperature regime of the soil and maintain its humidity [3, 6, 7]. Mulching of the soil with chicken manure showed good results in soil temperature and humidity and farmers can use this organic fertilizer as mulch when there are no other organic materials [8]. At the same time, mulch can have both positive and negative effects on crops [4, 5].

The aim of this study is to determine the impact of plant densities and soil mulching with different organic materials, on growth and yield in the cultivation of early spring lettuce.

II. MATERIAL AND METHOD

The experiment was conducted in 2014, in the experimental field of the University of Forestry – Sofia (42°7′ N, 23°43′E and 552 m altitude). The soil is fluvisol, slightly stony, slightly acidic. This area came under a continental climatic sub region, in a mountain climatic region.

The average daily air temperature during this period indicates that the months are cool, as a daily average temperature of the third ten days of May are suboptimal for the growth and development of lettuce. During the experiment, the monthly rainfall is abundant but not evenly distributed. Summary for April rainfall was 150,5 l / m2, and in May was 148,2 l / m2 (Fig. 1)

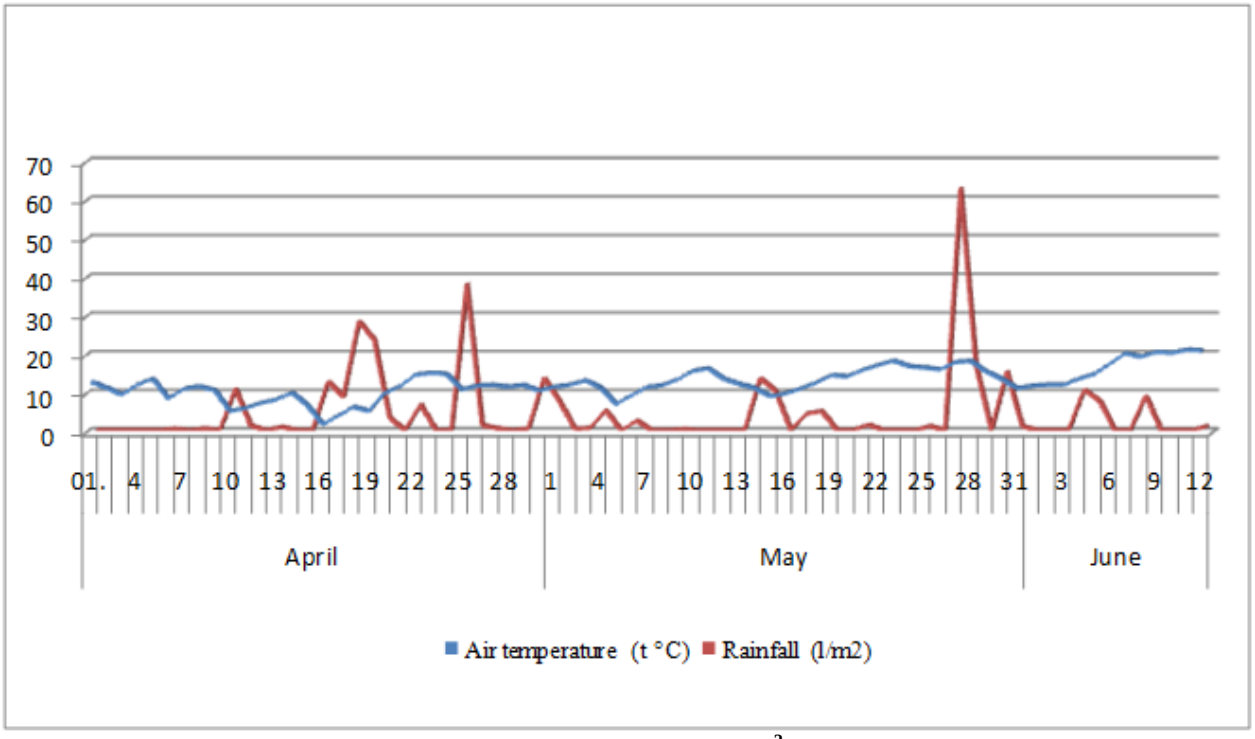

FIG. 1. AVERAGE AIR TEMPERATURE (t °C) AND RAINFALL (l/m^2) DURING THE EXPERIMENTAL PERIOD.

The study was performed with Romain lettuce (*Lactuca sativa* var. *romana*), cv. Yellow beauty, with growing period lasted 60-65 days, with pre-produced seedlings. Planting in the open field was carried out on 31th of March. All elements of agrotechnical activities (basic and pre-sowing cultivation, irrigation, etc.) were the same for all treatments. The plants were irrigated by drip irrigation system.

The experiment was designed with two different factors: A – plant density and B – soil mulcing. For Factor A the seedlings were planted at a distance of 30 x 30 cm and were tested two different schemes (Fig. 2): parallel planting to form a 4-row bed (R4) and a chess planting to form a 7-row bed (R7).

FIG. 2. TWO DIFFERENT SCHEMES OF CULTIVATION: PARALLEL PLANTING TO FORM A 4-ROW BED (R_4) AND A CHESS PLANTING TO FORM A 7-ROW BED (R_7)

For factor B were tested five treatments: bare soil with non-mulched and non-hoeing (weeded) control plot (NMC); mulch from barley straw (BSM); mulch from well-rotten horse manure (HMM); mulch from walnut wood sawdust (WSM); mulch from grass windrow (GWM).

The experiment was carried out by randomized complete block design with four replications and protection zones. The mulches were applied to the soil surface by hands at a thickness of 5-6 cm, after the seedlings of lettuce were strengthened. On the 60th day after planting was the beginning of harvesting. It was investigated plant height; plant diameter and plant weight. Data were obtained from 10 marked plants for each replication during harvesting period. The total yield is established in tones per hectar^{-1} (t/ha^{-1}) in replications and variants. Data were subjected to statistical analysis where was used an alpha level of .05. Test error rates were submitted to a two-way ANOVA. Differences between treatment means were compared with Fisher's Least Significant Differences (LSD).

III. RESULTS AND DISCUSSIONS

3.1 Main effect of plant density and mulching

Plant growth density has a significant impact on the biometric parameters and yields. In the seven-row bed (R_7), the plants have a bigger average height (24.65 cm) and a bigger average weight per plant (245 g), compared to the four-row bed (22.44 cm and 225 g). In contrast, the average plant diameter at the greater plant density is smaller (29.27 cm), compared to the average diameter (30.4 cm) of the four-row bed plants (Table 1). The total yield (3.572 t / ha^{-1}) at R7 compared to the total yield (1.872 t / ha^{-1}) is significantly higher (F (1.8) = 81.69, MSE = 0.088 p=.00002).

Soil mulching, as a single factor, also influences plant development and yield, and the impact is different depending on the type of used mulch. With the highest height (25.04 cm), a diameter (31.06 cm) and a weight per plant (273 g) are lettuces mulched with well-rotted horse manure. With the smallest diameter (28.95 cm) and the weight of one plant (211 g) are lettuces mulched with straw. At the smallest height (22.67 cm) are the lettuces mulched with weed sawdust, but in the comparison of the mean values by LSD test, there was no difference between them (Table 1). Of mulching variants, the total yield is highest (3,145 t / ha^{-1}) in the mulched plots with horse manure, and the lowest (2,421 t/ha^{-1}) in the straw mulch plots.

TABLE 1
MAIN EFFECT OF PLANT DENSITY AND MULCHING ON BIOMETRIC DATA AND PLANT YIELDS.

Factors	Height (cm)	Diameter (cm)	Weight per plant (g)	Total yield (t/ha^{-1})
A – No of rows per bed				
R4	22,44 b	30,40 a	225 b	1,872 b
R7	24,65 a	29,27 b	245 a	3,572 a
LSD	0,831	0,499	13,365	0,953
Significance level	0.001	0.001	0.01	0.001
B - mulches				
NMC	23,09 b	29,64 bc	224 bc	2,595 b
BSM	22,85 b	28,95 c	211 c	2,421 b
HMM	25,04 a	31,06 a	273 a	3,145 a
WSM	22,67 b	29,43 bc	236 b	2,784 ab
GWM	24,06 a	30,10 b	229 bc	2,665 ab
LSD	0,736	0,789	21,132	0,486
Significance level	0.05	0.05	0.05	0.05

values followed by different letters within the same column are significant different.

Greater plant density leads to an increase in their height, and this could be due to competition between plants, which confirms the results obtained by Alahi et al. (2014) and contrary to the data obtained from Moniruzzaman (2006). The greater average weight per plant is obtained at a higher plant density. Similar results are also obtained from Khazaei1 et al. (2013), but are contrary with the one obtained by Moniruzzaman (2006).

The lower plant density in the four-row bed leads to a larger plant diameter as it provides better space for their development, the significant increase in total yields in the seven-line bed is mainly due to the increased number of plants per unit area in chess planting. These data are in agreement with data obtained from Moniruzzaman (2006).

Horse manure is dark in color and improves soil temperature, especially in cool spring. It helps maintain a consistent soil moisture and is rich in nutrients. This leads to an increase in yields, similar to those obtained from Moreira et al. (2014) using poultry manure.

As mulching material the grass windrow covers well the soil, retains moisture, and while the lower layer decays it leads to the warming of the soil [16]. Sinkevičienė et al. (2009) found that mulching with grass windrow significantly increased the yield of onions, beetroot, cabbage and potatoes. A similar effect is caused by mulch of grass windrow on the growth of lettuce, especially in cooler spring.

Whereas straw is poor in nutrients, bright in color, reflects the sun's rays and does not help warming the soil. In a cooler spring, as it is in the experiment year, the straw maintains lower soil temperatures, which results to slow growth. Plants mulched with straw have a smaller height and diameter as well as with a lower weight than the plants of the other mulched variants and non-milled control. The mulching material of weed sawdust did not have a unique effect on the growth and development of the plants.

In general, mulching has had a positive effect on lettuce growth and on total yields, compared with non-mulching plots, except the straw mulch.

3.2 Combined effect of plant density and mulching

Fischer's test for the smallest difference between the mean values of the indicators revealed significant differences between the different combinations of the two factors. The use of horse manure as a mulching material, combined with plant density, has a significant effect on the growth of lettuce. In the variant with a combination of the mulch of horse manure and seven-row bed lettuces had the highest height and weight per plant. It is followed by the variant of the mulch from grass windrow in the same growing scheme. When combining these two mulching materials with a four-row bed, the plants had the largest diameter. The highest total yield of lettuce was obtained by combining horse manure mulch and seven-row bed (Table 2).

TABLE 2
COMBINED EFFECT OF PLANT DENSITY AND MULCHING ON BIOMETRIC DATA AND PLANT YIELDS.

Variants	Height (cm)	Diameter (cm)	Weight per plant (g)	Total yield (t/ha^{-1})
R_4NMC	21,79 f	30,53 bc	210 de	1,746 e
R_4BSM	22,22 ef	29,59 cd	207 e	1,725 e
R_4HMM	23,68 cd	31,66 a	269 a	2,240 d
R_4WSM	21,56 f	29,24 de	212 de	1,762 e
R_4GWM	22,94 de	30,99 ab	226 bcd	1,886 de
R_7NMC	24,39 bc	28,74 de	239 b	3,445 bc
R_7BSM	23,49 cd	28,31 e	215 cde	3,117 c
R_7HMM	26,40 a	30,46 bc	278 a	4,050 a
R_7WSM	23,78 cd	29,63 cd	261 a	3,805 ab
R_7GWM	25,18 b	29,21 de	232 bc	3,444 bc
$LSD_{0.05}$	1,041	1,116	16,677	0,434
CV%	6,45	3,52	11,06	34.49

values followed by different letters within the same column are significant different.

IV. CONCLUSION

From the results obtained in the experiment, it can be summarized that the use of horse manure and grass windrow, as a mulching material in cool spring, has a positive effect on lettuce development and in combination with a bigger plant density (seven-row bed) a higher yield is also obtained. In a cooler spring, the straw mulch leads to slow growth of the lettuce and low yield.

REFERENCES

[1] Alahi, A., Hossain, M. I., Kabir, K., Shahjahan, M., Arefin, S. M. A., & Hosain, M. T. (2014). Effect Of Phosphorus And Plant Spacing On The Growth And Yield Of Lettuce.. Adv. Agric. Biol.2 (1), 2014: 1-7

[2] Aminifard, M. H., Aroiee, H., Karimpour, S., & Nemati, H. (2010). Growth and yield characteristics of paprika pepper (Capsicum annum L.) in response to plant density. *Asian journal of plant sciences*, *9*(5), 276.

[3] Asaduzzaman, M. D., Shamima, S., & Arfan, M. D. (2010). Combined effect of mulch materials and organic manure on the growth and yield of lettuce. *J. Agric. Environ. Sci*, *9*, 504-508.

[4] Ji, S., & Unger, P. W. (2001). Soil water accumulation under different precipitation, potential evaporation, and straw mulch conditions. *Soil Science Society of America Journal*, *65*(2), 442-448.

[5] Kar, G., & Kumar, A. (2007). Effects of irrigation and straw mulch on water use and tuber yield of potato in eastern India. *Agricultural Water Management*, *94*(1), 109-116.

[6] Khazaei, I., Salehi, R., Kashi, A., & Mirjalili, S. M. (2013). Improvement of lettuce growth and yield with spacing, mulching and organic fertilizer. International Journal of Agriculture and Crop Sciences, 6(16), 1137.

[7] Moniruzzaman, M. (2006). Effects of plant spacing and mulching on yield and profitability of lettuce (Lactuca sativa L.). *Journal of Agriculture & Rural Development*, *4*(1), 107-111.

[8] Moreira, M. A., Santos, C. A. P. D., Lucas, A. A. T., Bianchini, F. G., Souza, I. M. D., & Viégas, P. R. A. (2014). Lettuce production according to different sources of organic matter and soil cover. Agricultural Sciences, Vol.5, No.2, 99-105

[9] Sadeghi, S., Rahnavard, A., & Ashrafi, Z. Y. (2009). The effect of plant density and sowing date on yield of Basil (Ocimum basilicum L.) in Iran. *Journal of Agricultural Technology*, *5*(2), 413-422.

[10] Sinkevičienė, A., Jodaugienė, D., Pupalienė, R., & Urbonienė, M. (2009). The influence of organic mulches on soil properties and crop yield. *Agronomy Research*, *7*(1), 485-491.

[11] Siwek, P., Kalisz, A., & Wojciechowska, R. (2007). Effect of mulching with film of different colours made from original and recycled polyethylene on the yield of butterhead lettuce and celery. *Folia Hort. Supl*, *19*(1), 25-35.

[12] Schonbeck, M. W., & Evanylo, G. K. (1998). Effects of mulches on soil properties and tomato production I. Soil temperature, soil moisture and marketable yield. Journal of Sustainable Agriculture, 13(1), 55-81.

[13] Schonbeck, M. W., & Evanylo, G. K. (1998). Effects of mulches on soil properties and tomato production II. Plant-available nitrogen, organic matter input, and tilth-related properties. Journal of Sustainable Agriculture, 13(1), 83-100.

[14] Schonbeck, M. W. (1999). Weed suppression and labor costs associated with organic, plastic, and paper mulches in small-scale vegetable production. Journal of Sustainable Agriculture, 13(2), 13-33.

[15] Yordanova, M., N. Gerasimova, 2012. Effect of mulching on soil temperature in growing onions chapter (Allium cepa L.) Ecology and Health, Proceedings of the ninth national scientific conference with international participation. Academic Publishing Agricultural University. Plovdiv. 165-170

[16] Yordanova, M., & Gerasimova, N. (2015). Influence of different organic mulches on soil temperature during pepper (Capsicum annuum L.) cultivation. Scientific Papers-Series B, Horticulture, (59), 285-290.

Solar Irradiance Forecasting Using Intelligent Technology

Yi-Ju Chang[1], Ko-Ming Mu[2], Yih-Guang Leu[3*], Li-Fen Chou[4]

[1.2.3]Department of Electrical Engineering, National Taiwan Normal University, Taiwan
[4]Taiwan Power Research Institute, Taiwan Power Company, Taiwan
*Email: leuyk@ntnu.edu.tw (corresponding author)

Abstract*—Because solar power is susceptible to clouds and substances in the air, the solar photovoltaic cannot produce stable power output. Solar irradiance is a measurement of the power output of photovoltaic module. Therefore, this paper uses some different combination inputs of the neural network to develop the solar irradiance forecasting with 24 hours ahead. Their forecasting performances are evaluated and some comparison results in Taichung solar farm are given.*

Keywords—Neural network, Solar Irradiance, Solar Power Systems.

I. INTRODUCTION

To solve the problems of limited fossil fuels and their impact on the environment, renewable resources play an important role. Solar energy is a very important renewable energy. Based on evaluated condition of solar power, solar photovoltaic becomes the most potential renewable energy in Taiwan. Solar irradiance is a measurement of the power output of photovoltaic module. However, because solar irradiance is influenced by substances in the air, the solar photovoltaic cannot produce stable power output. The power output of photovoltaic module is influenced immediately when the module is sheltered from the clouds. Besides, the material of solar cell, air temperature, module's position and orientation also affect the power output of the photovoltaic module. Therefore, it is an important issue to forecast solar irradiance accurately. Forecasting accuracy is not only influenced by the change of weather but also surroundings and the effectiveness of method and data. Developing an excellent solar power systems not only can wield the change of solar power but also help power company to allocate power. The accuracy of solar irradiance forecasting is the basis of solar power forecasting [1-6]. There are many intelligent approaches to forecast solar irradiance, such as neural networks [7-13].

The paper uses neural network technology to develop the solar power forecasting with 1-24 hours ahead. Some different features for solar forecasting are proposed and their forecasting performances are evaluated. Moreover, comparison results in Taichung solar farm in Taiwan [14] are given.

II. METHODS OF SOLAR IRRADIANCE FORECASTING

The backpropagation (BP) neural network is used in this paper, and the main structure of which is input layer, hidden layer, and output layer. Input layer transfer received external information into the network, and which is used to represent the input variable. Hidden layer process the input data, which is used to represent the relationship between input variables. Input data are transferred to the output layer after being converted by nonlinear transform function. Output layer receives and outputs the information from upper layer, and is used to represent the output variable of network. The layers connect to each other to deliver information, and this connection is called "weight". The weight value is acquired from the iterative learning calculation of the neuro. Using supervised learning method is to reduce the discrepancy between output and expected value, and to minimize the performance index of the network. The training method in this paper is Levenberg-Marquardt algorithm, which is a modified gradient descent method and possesses the ability of fast training.

Based on the assumption that there is no cloud and shelter, solar irradiance can be calculated as follows [15].

A. The air mass is defined as follows.

$$\mathrm{AM} = \frac{1}{\cos\zeta} \tag{1}$$

where ζ is zenith angle.

B. The intensity of the direct component of sunlight in units of kW/m^2 on the assumption that the location height above sea level is zero is defined as follows.

$$I_D = 1.353 \cdot 0.7^{AM^{0.678}} \tag{2}$$

where the value 1.353 is solar constant, the value 0.7 means that solar irradiance irradiates to the earth's surface through atmosphere is 0.7 times of itself, and the value 0.678 is empiric value.

C. The global solar irradiance is defined as follows.

$$I_G = 1.1 \cdot I_D \tag{3}$$

where the diffuse radiation is about 10% of the direct component of sunlight.

Root Mean Square Error (RMSE) can show the difference between the forecasting values and observed values, judge the quality of the neural network, and judge the level of convergence in the process. RMSE is closely related to the number of training data. The larger the RMSE, the greater the difference of the forecasting values and observed values may be. The closer the difference between RMSE and 0, the closer the difference between the forecasting values and observed values may be. RMSE is defined as follows.

$$RMSE = \sqrt{\frac{1}{n}\sum_{j=1}^{n}\left(Y_j - \hat{Y}_j\right)^2} \tag{4}$$

where Y_j is forecasting values , $\hat{Y}_j$ is observed values.

Based on some different combination inputs of the neural network, this paper proposes six methods to develop the solar irradiance forecasting with 24 hours ahead. The method 1 is to use the historical solar irradiance values in the daytime. The method 2 is to use the historical solar irradiance values in the daytime and the temperature. The method 3 is to use the historical solar irradiance values in the daytime and the solar altitude. The method 4 is to use the historical solar irradiance values in the daytime, the temperature and the solar altitude. The method 5 is to use the difference value of historical solar irradiance values and the solar altitude. The method 6 is to use the historical solar irradiance values and the future solar irradiance value that is calculated on the assumption that there is no cloud and shelter [15]. The training data in Taichung solar farm in Taiwan [14] were in the period from March 2014 to September 2015. In the methods 1-6, the values of solar irradiance next hour is the only output. The data of the network training only apply the daytime data. Defining the day or night is depending on the calculated values of solar altitude [15]. If the solar altitude is larger than zero, it is in the daytime. Conversely, it is at night.

In the method 1, the architecture of back propagation neural network was made from 3 inputs, 7 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 3 inputs are the observed value of solar irradiance at the time *t*, the time *t*-1, the time *t*-2.

In the method 2, the architecture of back propagation neural network was made from 4 inputs, 9 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 4 inputs are the observed value of solar irradiance at the time *t*, the time *t*-1, the time *t*-2 and the temperature.

In the method 3, the architecture of back propagation neural network was made from 4 inputs, 11 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 4 inputs are the solar altitude, the calculated value of solar irradiance, the observed value of solar irradiance at the time *t*, and the time *t*-1.

In the method 4, the architecture of back propagation neural network was made from 5 inputs, 10 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 5 inputs are the solar altitude, the temperature, the calculated value of solar irradiance, the observed value of solar irradiance at the time *t*, and the time *t*-1.

In the method 5, the architecture of back propagation neural network was made from 3 inputs, 8 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 3 inputs are the solar

altitude, the difference value of calculated value and observed value of solar irradiance, and the difference values of solar irradiance at the time *t*, and the time *t*-1.

In the method 6, the architecture of back propagation neural network was made from 4 inputs, 7 hidden layer neurons and 1 output. The transition function of hidden layer neurons is on-linear hyperbolic tangent function. 4 inputs are the calculated value of solar irradiance next hour, the observed value of solar irradiance at the time *t*, the time *t*-1 and the time *t*-2.

In order to judge the quality of the forecasting, the RMSEs of the methods 1-6 are calculated by equation (4). The RMSEs of the methods 1-6 are shown in Figs.1-5. The results of RMSE comparison for the solar irradiance forecasting with 1, 3, 6, 12 and 24 hours ahead are shown in Figs.1-5, respectively.

According to comparison for the values of RMSE, neural networks combined with future solar irradiance have the lowest value of RMSE.

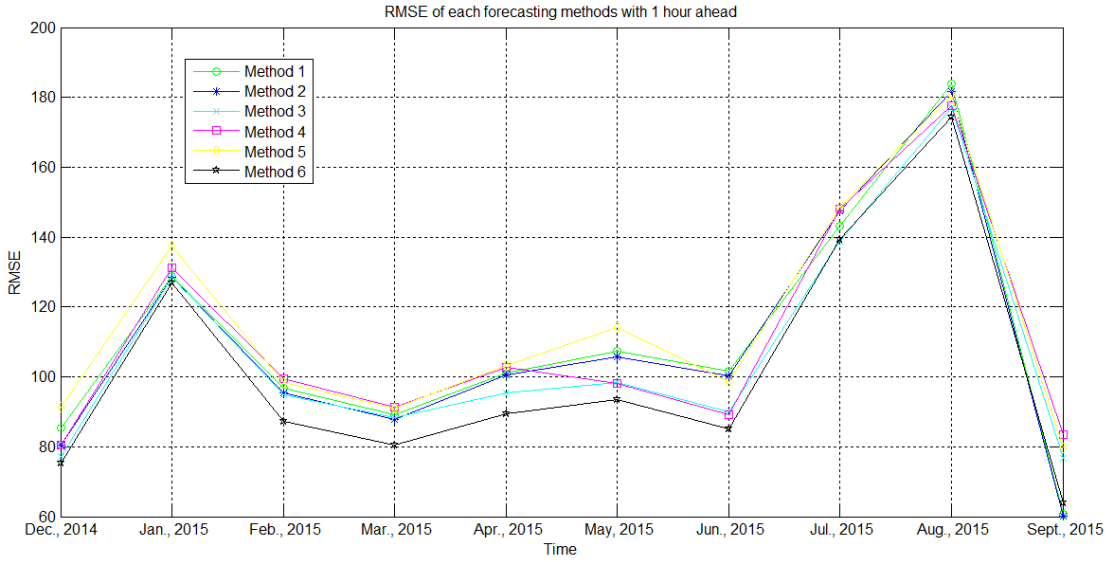

FIG.1. RMSE of each forecasting methods with 1 hour ahead.

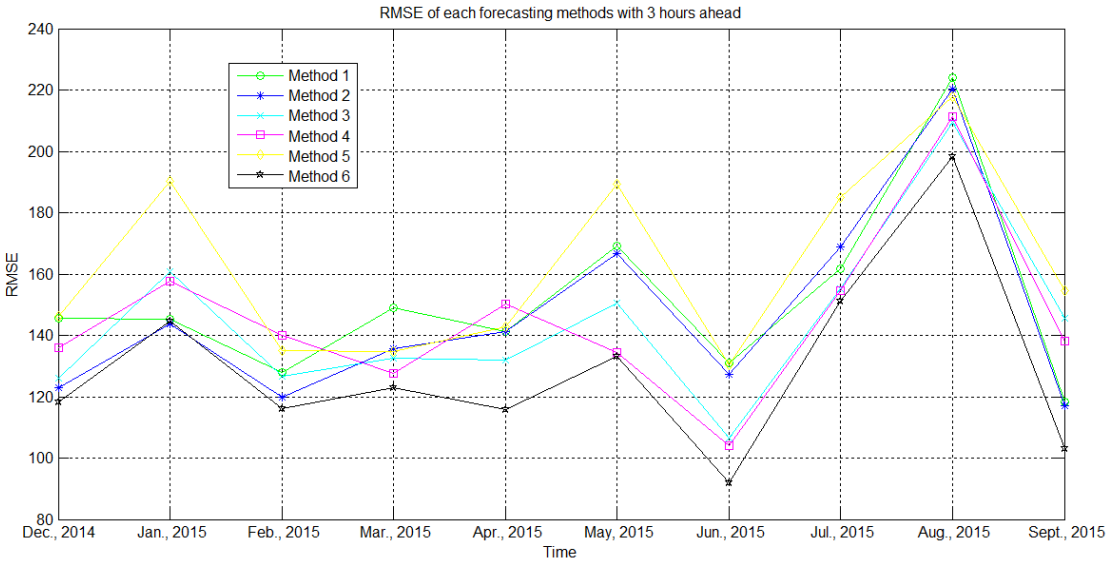

FIG.2. RMSE of each forecasting methods with 3 hours ahead.

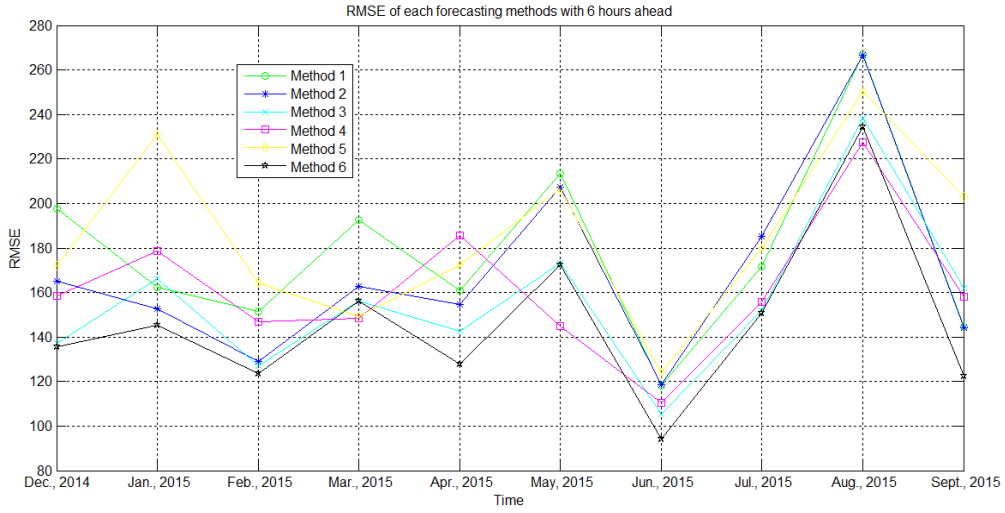

FIG.3. RMSE of each forecasting methods with 6 hours ahead.

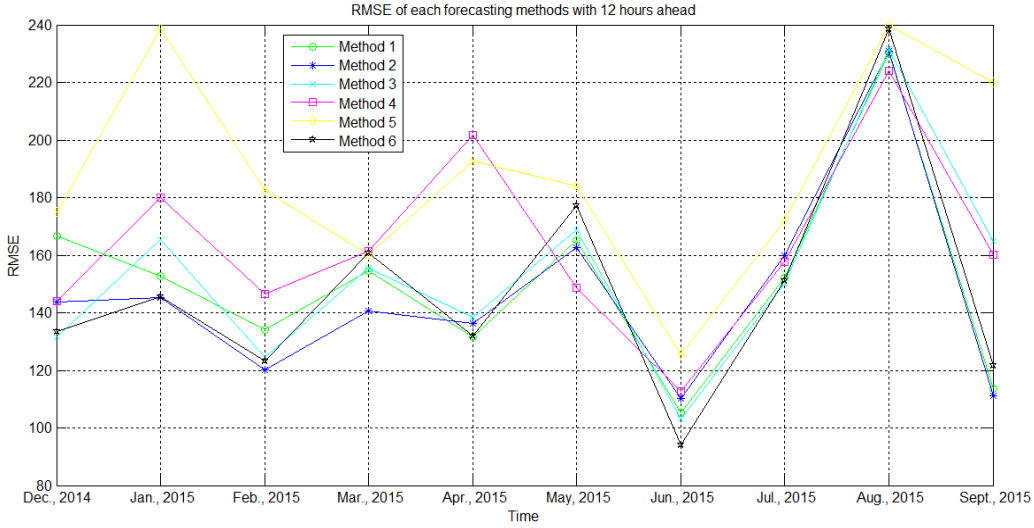

FIG.4. RMSE of each forecasting methods with 12 hours ahead.

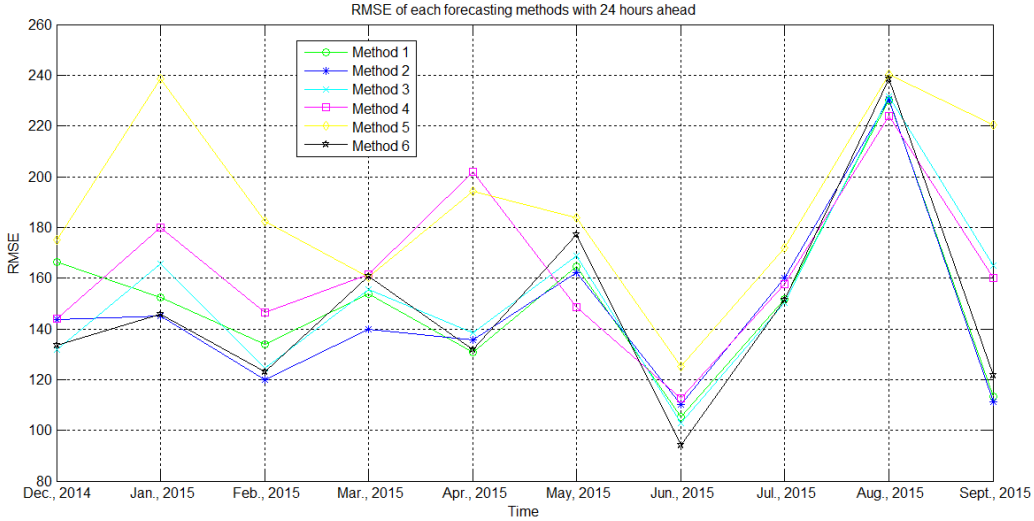

FIG.5. RMSE of each forecasting methods with 24 hours ahead.

III. CONCLUSION

This paper has proposed some different features for solar irradiance forecasting and presented some comparison results of solar irradiance forecasting in Taichung solar farm. According to the RMSE comparison figures, the method depend on historical and future solar irradiance values is better than other methods in forecasting with 1-24 hours ahead. Moreover, the results are likely to be affected by the historical data in the forecasting with short-time ahead.

ACKNOWLEDGEMENTS

This work was supported by the Taiwan Power Company, R.O.C. and the National Science Council of Taiwan, R.O.C., under Grants NSC 102-2221-E-003-011.

REFERENCES

[1] R. Perez, M. Beauharnois, E. Lorenz, S. Pelland, and J. Schlemmer, "Evaluation of numerical weather prediction solar irradiance forecasts in the US," in Proc. ASES Annual Conference, Raleigh, NC, USA, May. 2011.

[2] P. Mathiesen and J. Kleissl, "Evaluation of numerical weather prediction for intra-day solar forecasting in the continental United States," Solar Energy, vol. 85, pp.967-977, 2011.

[3] E. Lorenz, J. Hurka, D. Heinemann, and HG. Hans,"Irradiance Forecasting for the Power Prediction of Grid-Connected Photovoltaic Systems," IEEE Journal of Selected Topics in Applied Earth Observations and Remote Sensing, vol. 2, no. 1, Mar. 2009.

[4] P. Bacher, H. Madsen, and H.A. Nielsen, "Online short-term solar power forecasting," Solar Energy, vol. 83, pp.1772-1783, 2009.

[5] A. Hammer, D. Heinemann, E. Lorenz, and B. Luckehe, "Short-term forecasting of solar radiation: a statistical approach using satellite data," Solar Energy, vol. 67, No.1-3, pp.139-150, 1999.

[6] C.W. Chow, B. Urquhart, M. Lave, A. Dominguez, J. Kleissl, J. Shields, and B. Washom, "Intra-hour forecasting with a total sky imager at the UC San Diego solar energy testbed," Solar Energy, vol. 85, pp.2881-2893, 2011.

[7] F. Wang, Z. Mi, S. Su and H. Zhao,"Short-Term Solar Irradiance Forecasting Model Based on Artificial Neural Network Using Statistical Feature Parameters", Energies, vol.5, pp.1355-1370, 2012.

[8] A. Mellit and A. M.Pavan, "A 24-h forecast of solar irradiance using artificial neural network: Application for performance prediction of a grid-connected PV plant at Trieste, Italy", Solar Energy, vol. 84, pp.807-821, 2010.

[9] A. Yona, T. Senjyu, A.Y. Saber, F. Toshihisa, S. Hideomi and K. Chul-Hwan,"Application of Neural Network to One-Day-Ahead 24 hours Generating Power Forecasting for Photovoltaic System", IEEE Intelligent Systems Applications to Power Systems , Niigata, pp.1-6, 2007.

[10] J. Cao and X. Lin,"Application of the diagonal recurrent wavelet neural network to solar irradiation forecast assisted with fuzzy technique," Engineering Applications of Artificial Intelligence, vol. 21, pp.1255-1263, 2008.

[11] A. Mellit, M. Benghanem, and S.A. Kalogirou, "An adaptive wavelet-network model for forecasting daily total solar-radiation," Applied Energy, vol. 83, pp.705-722, 2006.

[12] C. Paoli, C. Voyant, M. Muselli, and M.-L. Nivet, "Forecasting of Preprocessed Daily Solar Radiation Time Series Using Neural Networks," Solar Energy, vol. 84, no. 12, pp. 2146–2160, Dec. 2010.

[13] F. Gutierrez-Coreaab, M. Manso-Callejoa, M. Moreno-Regidora, and M. Manrique-Sanchoa ,"Forecasting short-term solar irradiance based on artificial neural networks and data from neighboring meteorological stations," Solar Energy, vol.134, pp. 119-131, 2016.

[14] http://www.taipower.com.tw/content/new_info/new_info-b31.aspx?LinkID=8, 2017.

[15] http://www.pveducation.org/, 2017.

Productivity Assessment of Drought Tolerant Rice Cultivars under Different Crop Management Practices in Central Terai of Nepal

Bishal Dhakal[1*], Samjhana Khanal[2], Lal Prasad Amgain[3]

[1]Regional Agricultural Research Station (RARS), Bhagetada, Dipayal, Doti, Nepal
[2,3]Institute of Agriculture and Animal Science (IAAS), Sundarbazar, Lamjung, Nepal
*Corresponding author: dhakal.bishal14@gmail.com

***Abstract*—** *Proper selections of resource conservation technologies and drought tolerant cultivars are being potential strategies determining productivity of rice in drought prone areas. Thus, a field experiment was accomplished in central-terai of Nepal during 2014 to assess the productivity of drought tolerant rice cultivars under different crop management practices. The experiment was carried out in strip-plot design with three replications consisting four drought tolerant rice cultivars and three crop management practices. The analyzed data revealed that SRI (System of Rice Intensification) produced significantly higher grain yield (5.28 t ha^{-1}) than other management practices. The straw yield of SRI (5.12 t ha^{-1}) was also significantly higher than other management practices. The cultivars had no influence on grain yield, but the straw yield was significantly influenced by cultivars, with the highest straw yield in Sukkha-3 (5.21 t ha^{-1}). Thus, SRI management practice can be adopted as adaptation approach for obtaining higher productivity in central terai and similar agro-climatic regions of Nepal.*

***Keywords*— Crop management practices, Productivity, Rice, SRI.**

I. INTRODUCTION

Rice is the second most important staple food for more than half of the world's population [1, 2]. Being a most important staple food of Nepalese people, rice ranks first crop for both acreage and production and production amounts to half of the total cereal grains in the country [3]. In Nepal, rice is grown in about 1.42 million hectares with total production about 4.50 million tons, and 3.17 t ha^{-1} productivity [4]. The share of agriculture and forestry for national gross domestic product (GDP) is 33.03%, and therein rice alone contributes 20.75% of the agriculture gross domestic product (AGDP) and 10.2% of total GDP [5].

In Nepal, more than 70% of the total rice area is grown under rainfed condition [6], whereas only 21 % rice production is under partially or fully irrigated conditions [7]. Rice production relies on ample water supply and thus is more vulnerable to drought stress than other crop. The temperature of Nepal has increased by 0.04-0.06 ºC annually on an average during 1977-2005 [8]. Increase in temperature due to climate change has resulted an increase in evidences of drought stress in crop production including rice [9]. According to statistics, the percentage of drought affected lands areas more than doubled from the 1970s to the early 2000s worldwide [10]. Further, increased temperature may decrease rice potential yield up to 7.4% per degree increment of temperature [11]. Several other factors like weeds, low factor productivity and reducing resource use-efficiency due to deteriorating soil health are causing the lower productivity of rice in Nepal. Among various approaches to climate change adaptation in drought prone areas, proper selections of resource conservation technologies like (SRI, ICM, etc.) [12] and drought tolerant rice cultivars [13] are potential strategies determining yield of rice. Thus, the present investigation is planned, executed and accomplished with the objective of pursuing the productivity of various drought tolerant rice cultivars under different crop management practices in central terai of Nepal.

II. MATERIALS AND METHODS

This study was carried out at Dhauwadi VDC, Nawalparasi (235 masl) from June to October 2014. The experimental site is situated at 27°48'43" N latitude and 84°4'58" E longitude, where it received 1045 mm of rainfall during the experimental period. The experiment was carried out using a strip plot design, in the fields of three farmers, considering each farmer as a replication. The treatment consists of combination of the column factor (three rice management practices: System of Rice Intensification-SRI, Integrated Crop Management-ICM and Puddled transplanted-conventional) and row factor (four rice

cultivars: Sukkha-3, Sukkha-4, Sukkha-5 and Hardinath-2). The size of each plot was 12 m^2, and the net plot was determined after leaving one border row in each side, one destructive sampling row and one guard row. The space between two plots was 0.5 m, and the bund of 0.5 m was made between each management practices to check the flow of water and nutrients between them. The experiment on three management practices were set up considering the production factors (Table 1). Vermicompost was used as a source of organic manure, whereas Urea, DAP and MOP were used as sources of N, P_2O_5 and K_2O, respectively. Full doses of phosphorus and potassium and half dose of nitrogen were applied as basal dose at the time of transplanting. The remaining half dose of nitrogen was applied in two split doses: one-fourth N at 30 DAT and the remaining one-fourth at booting stage. The crop from net plot area was harvested manually with the help of sickles. The whole plant was cut at 2 cm above ground for all varieties, except Hardinath-2 that was harvested by hand picking of panicles due to heavy rainfall during harvesting period. The grains were weighted at their exact moisture content and were adjusted at 14% moisture level. The biometric observations (plant height, tillers number per square meter, LAI, above ground dry matter), yield attributing characters and yields of all the treatments were recorded. These recorded data's were tabulated in MS-Excel which was subjected to ANOVA [14], after analysis through MSTAT-C and mean separation for significant variables were done by Duncan's Multiple Range Test (DMRT) at 5% level of significance.

TABLE 1
PRODUCTION FACTORS CONSIDERED IN DIFFERENT MANAGEMENT PRACTICES

Production factors	SRI	ICM	Conventional
Crop geometry	25 cm × 25 cm	20 cm × 20 cm	20 cm × 15 cm
Seed rate	7.5 kg ha^{-1}	20 kg ha^{-1}	40 kg ha^{-1}
Seedling age	14 days old	21 days old	28 days old
Seedling/hill	1	2	3
Organic manure	10 t ha^{-1}	5 t ha^{-1}	None
NPK	20:15:10 kg ha^{-1}	40:30:20 kg ha^{-1}	80:60:40 kg ha^{-1}
Water management	Alternating wetting and drying	Intermediate condition	Flooded condition

III. RESULTS AND DISCUSSIONS

3.1 Grain yield

The grain yield was significantly influenced by management practices, but the cultivars and its interaction with management practices had no influence on grain yield (Table 2). The grain yield of SRI management practice (5.28 t ha^{-1}) was significantly higher than conventional management practice (4.49 t ha^{-1}), but it was statistically at par with ICM management practice (4.73 t ha^{-1}). The grain yield of ICM practice was also significantly higher than conventional (228 m^{-2}) management practice. The higher grain yield of SRI management practice was because of significantly higher number of effective tillers (318 m^{-2}) than ICM (387 m^{-2}) and conventional management practices. Panicle weight, panicle length and filled grains per panicle of SRI management practice were also significantly higher than ICM and conventional management practices. Further, sterility percentage was significantly lower in SRI (14.97%) than ICM (15.13%) and conventional (16.23%) management practices. Further, it was revealed that there was positive correlation between grain yield and effective tillers per square meter (r= 0.75) (Figure 1), filled grain per panicle (r= 0.91) (Figure 2), panicle length (r=0.82) (Figure 3) and panicle weight (r=0.71) (Figure 4). Higher number of effective tillers, panicle weight and filled grains per panicle were reported in SRI than conventional management practice [15],[16],[17],[18]. The higher grain yield of SRI was also due to higher LAI as compared to other management practices. The grain yield of rice is also determined by assimilates deposited mainly in vegetative stage, which is directly contributed by leaf area. Carbohydrates produced before heading mainly accumulate in the leaf sheath and stem and translocate to the panicles during grain filling [19]. The contribution of carbohydrates produced before heading to the final grain yield appeared to be in range of 20-40 % [20].

TABLE 2

GRAIN YIELD, STRAW YIELD AND HARVEST INDEX OF VARIOUS CULTIVARS OF RICE AS AFFECTED BY MANAGEMENT PRACTICES AT DHAUWADI VDC, NAWALPARASI, NEPAL, 2014

Treatment	Grain yield (t ha^{-1})	Straw yield (t ha^{-1})	Harvest Index (%)
Management			
SRI	5.28 ^{a}	5.12 ^{a}	46.96
ICM	4.73 ^{ab}	4.73 ^{b}	46.14
CON	4.49 ^{b}	4.06 ^{c}	49.02
SEm (±)	0.145	0.057	0.885
LSD (0.05)	0.57	0.23	ns
Cultivars			
Sukkha-3	4.79	5.21 ^{a}	44.06
Sukkha-4	4.73	4.43 ^{b}	47.94
Sukkha-5	5.16	4.49 ^{b}	50.02
Hardinath-2	4.64	4.42 ^{b}	47.48
SEm (±)	0.236	0.108	1.30
LSD (0.05)	ns	0.37	ns
CV (%)	10.81	5.1	6.7
Grand Mean	4.83	4.64	47.37

(Treatment means followed by common letter/letters within column are not significantly different among each other based on DMRT at 0.05; **= significant at 0.01 level, *= significant at 0.05 level and ns= non-significant at 0.05 level)

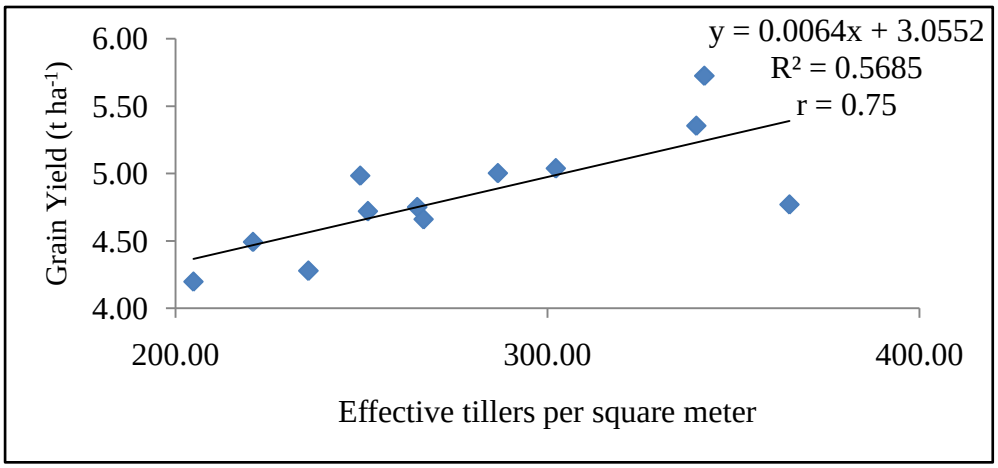

FIGURE 1: LINEAR REGRESSION BETWEEN GRAIN YIELD AND EFFECTIVE TILLERS PER SQUARE METER

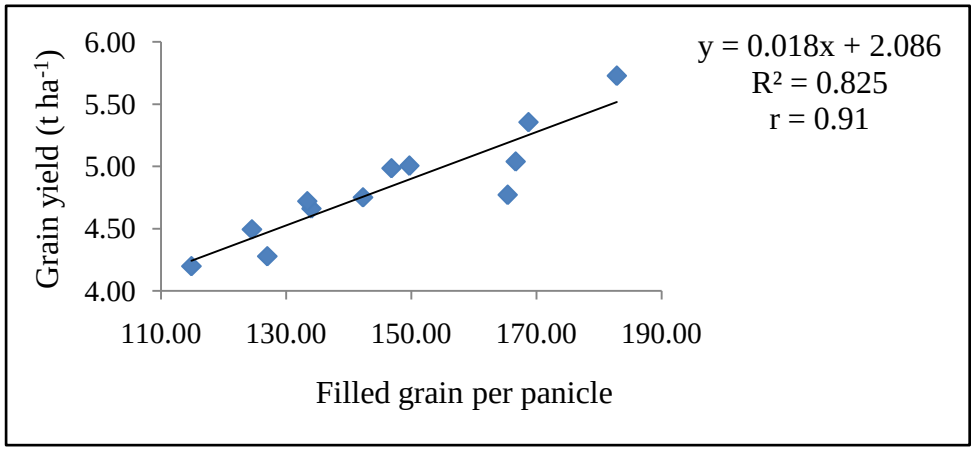

FIGURE 2: LINEAR REGRESSION BETWEEN GRAIN YIELD AND FILLED GRAIN PER PANICLE

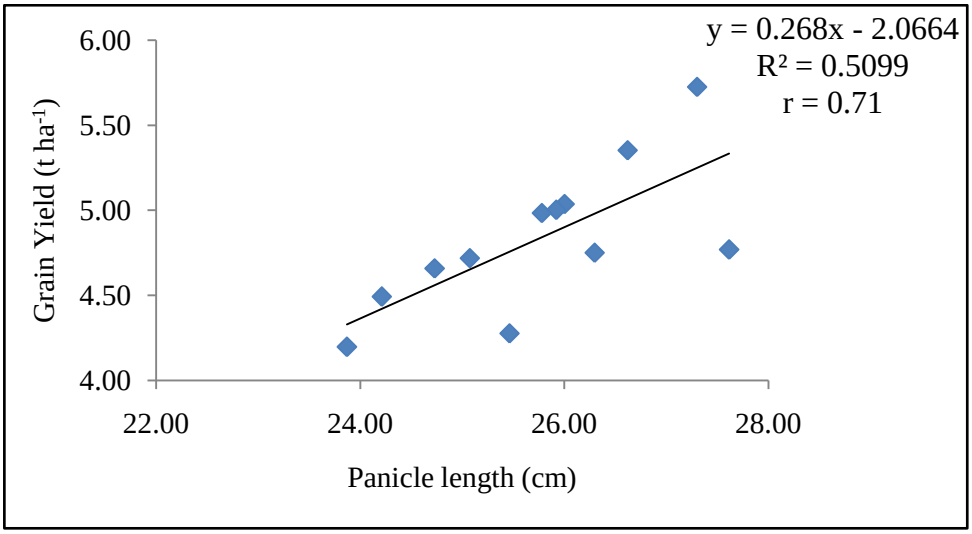

FIGURE 3: LINEAR REGRESSION BETWEEN GRAIN YIELD AND PER PANICLE LENGTH

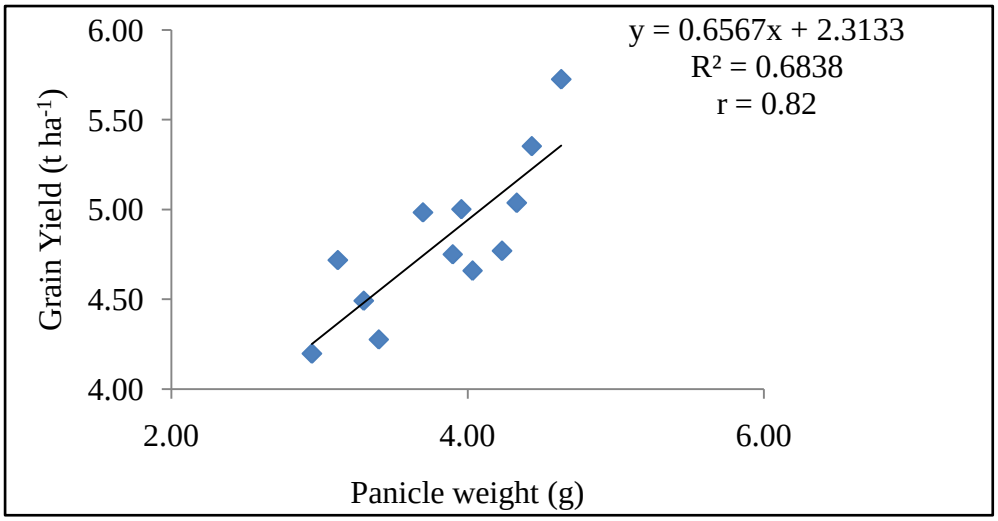

FIGURE 4: LINEAR REGRESSION BETWEEN GRAIN YIELD AND PER PANICLE WEIGHT

It was revealed that SRI practice produced 17.49% more yield than conventional practice. Although SRI and ICM practices were statistically similar, SRI produced 11.63% more yield than ICM practice. Moreover, ICM produced 5.35 % more grain yield as compared to conventional management practice. The increase in grain yield of 11.8 % was reported under SRI management practice over conventional [21]. Similarly, increase in grain yield under SRI and ICM management practices was 209.9 % and 185.4 % higher, respectively over conventional management practices [16]. Moreover, 100-200 % increase in grain yield was also reported under SRI compared to conventional management practice [22].

3.2 Straw yield

The straw yield (5.12 t ha^{-1}) of SRI practice was significantly higher than ICM (4.73 t ha^{-1}) and conventional practices (4.06 t ha^{-1}). The straw yield of ICM practice was also significantly higher than conventional practice. This might be due to longer plant height in SRI and ICM management practices over conventional management practices. Moreover, early vigorous growth due to wider spacing which resulted less competition in space, nutrition and other factors for growth might have resulted higher straw yield in SRI management practice. Further, the higher straw yield in SRI might also be due to higher number of tillers in SRI than other management practices [23]. The significant higher straw yield in SRI than in conventional management practices was also reported in earlier experiments [23], [24].

The straw yield of Sukkha-3 (5.21 t ha^{-1}) was significantly higher than other varieties, whereas the straw yield of other cultivars were at par (Table 2). Higher straw yield of Sukkha-3 might be due to longer plant height of this cultivar. Higher straw yield in the cultivars with longer plant height was also reported in earlier experiment [25]. Higher dry matter accumulation in Sukkha-3 might also had contributed to its higher straw yield. Further, there was significant influence of interaction of cultivars and management practices in straw yield. The mean straw yield was found highest in Sukkha-5 with

SRI (5.66 t ha^{-1}), followed by Sukkha-3 with ICM practices (5.31 t ha^{-1}). The lowest mean straw yield (3.56 t ha^{-1}) was observed in Sukkha-5 with conventional practice.

3.3 Harvest Index

The harvest indexes of all the management practices were statistically at par. Similarly, the cultivars also had no any significant influence on harvest index.

IV. CONCLUSION

The results showed that grain yield was significantly influenced by management practices, where SRI management practice recorded the highest grain yield than other management practices. But, the rice cultivars and the interaction of management practices and cultivars had no influence on grain yield. Thus, SRI management practice can be adopted as adaptation approach for obtaining higher grain yield in central terai and similar agro-climatic regions of Nepal.

ACKNOWLEDGEMENT

The authors are thankful to SAFBIN project of CARITAS-Nepal for providing financial support for the carrying out the field experiment. Similarly, farmers of experiment sites are also highly acknowledged for their support during the experimental period.

REFERENCES

[1] Delseny, M.; Salses, J.; Cooke, R.; Sallaud, C.; Regad, F.; Lagoda, P.; Guiderdoni, E.; Ventelon, M.; Brugidou, C.; Ghesquière, A. Rice genomics: Present and future. Plant Physiol. Biochem. 2001, 39(3-4), 323–334, doi: 10.1016/S0981-9428(01)01245-1

[2] Feng, Y.; Zhai, R.R.; Lin, Z.C.; Cao, L.Y.; Wei, X.H.; Cheng, S.H. QTL analysis for yield traits in rice under two nitrogen levels. Chin J. Rice Sci. 2013, 27(6), 577–584 (in chinese with English abstract), doi: 10.3969/j.issn.1001-7216.2013.06.003

[3] Ghimire, S.; Dhungana, S.M.; Krishna, V.; Teufel, N.; Sherchan, D.P. Biophysical and socio-economic characterization of cereal production systems of Central Nepal. Socioeconomics Program Working Paper 9. 2013. Mexico, D.F., CIMMYT. ISBN: 978-607-8263-21-9

[4] MoAD. Statistical information on Nepalese agriculture (2012/2013). Government of Nepal. Ministry of Agricultural Development. Agribusiness Promotion and Statistics Division. Singha Durbar, Kathmandu, 2013.

[5] Poudel, M.N. Rice (*Oryza sativa* L.) cultivation in the highest elevation of the world. Agron. JN 2011, 2, 31-41, doi: 10.3126/ajn.v2i0.7519

[6] CBS. Statistical year book of Nepal. HMG National Planning Commision Secretariat, Central Bureau of Statistics, Kathmandu, Nepal, 2003, p. 214.

[7] NARC. Research Highlights: 2002/03-2006/07. Communication, Publication and Documentation Division, Nepal Agricultural Research Council, Khumaltar. Lalitpur, Nepal, 2008; pp. 1-13.

[8] MoE. National Adaptation Programme of Action (NAPA). Government of Nepal, Ministry of Environment (MoE), Kathmandu, Nepal, 2010, p. 8.

[9] Karn, P.K. The impact of climate change on rice production in Nepal. South Asian Network for Development and Environmental Economics (SANDEE) Working Paper, 2014, 85-14:1-24, ISBN: 978-9937-596-15-2.

[10] Isendahl, N.; Schmidt. G. Drought in the Mediterranean: WWF policy proposals. WWF Report, Madrid 2006, pp 8

[11] Murdiyarso, D. Adaptation to Climatic Variability and Change: Asian Perspectives on Agriculture and Food Security. Environ. Monit. Assess. 2000, 61(1), 123-131. doi:10.1023/A:1006326404156

[12] Islam, M.; Nath, L.K; Das, A.; Smajdar, T. Productivity and Economic Performance of *Sali* rice under system of rice intensification and integrated crop management as influenced by weed management practices. Indian J. Hill Fmg. 2014, 27(1), 184-192.

[13] Basnet, B.M.S. National rice day: Rice and food security. Gorkhapatra online.com, November 5, 2015.

[14] Gomez, K.A; Gomez, A.A. Statistical procedures for agricultural research, 2nd ed.; John Wiley and Sons, New York, 1984, p. 108, ISBN: 978-0-471-87092-0

[15] Rao, A.U.; Ramana, A.V.; Sridhar, T.V. 2013. Performance of system of rice intensification (SRI) in Godavari delta of Andhra Pradesh. Ann Agric. Res. 2013, 34(2), 118-121.

[16] Islam, M.; Nath, L.K.; Patel, D.P.; Das, A.; Munda, G.C.; Samajdar, T.; Ngachan, S.V. Productivity and socio-economic impact of system of rice intensification and integrated crop management over conventional methods of rice establishment in eastern Himalayas, India. Paddy Water Environ. 2014, 12, 193-202, doi : 10.1007/s10333-013-0377-z

[17] Ahmed, A.R.; Dutta, B.K.; Ray, D.C. Response of some rice varieties to different crop management practices towards morphological and yield parameters. IJSRP. 2015, 5(2), 6. ISSN 2250-3153

[18] Jana, K.; Mallick, G.K.; Ghosh, S.; Sardar, G. Study on yield potentiality and spatial requirement of rice varieties (*Oryza sativa* L.) in system of rice intensification (SRI) under red and laterite zone of West Bengal, India. J. Appl. Nat. Sci. 2015, 7(1), 353-357, ISSN : 0974-9411 (Print), 2231-5209 (Online)

[19] Fageria, N.K. Yield physiology of rice. J. Plant Nutr. 2007, 30, 843-879. doi: 10.1080/15226510701374831

[20] Murata, Y. and S. Matsushima. Rice. In: Crop physiology: Some case histories. Evans, T., ed.; Cambridge University Press, London, 1975, Pp. 73-99, *ISBN*-10: 0521204224

[21] Gulshan, M.; Sarao, P.S. Evaluation of system of rice (*Oryza sativa* L.) intensification (SRI) in irrigated agro-ecosystem of Punjab. J. Res-ANGRAU, 2009, 37, 1-6.

[22] Munda, G.C.; Ngachan, S.V.; Das, A.; Malngiang, S.; Chowdhury, S. Site specific farming system options for rural livelihood-success stories from NEH Region. NAIP Bulletin, ICAR Research Complex for NEH Region, Umiam, 2012, 2, 1-78.

[23] Wijebandara, D.M.D.I.; Dasog, G.S.; Patil, P.L.; Hebbar, M.. Effect of nutrient levels and biofertilizer on growth and yield of paddy under system of rice intensification (SRI) and conventional methods of cultivation. Tropical Agricultural Research, 2008, 20, 87-97.

[24] Jeyapandiyan, N.; Lakshmanan, A. Yield comparison of rice in different cultivation systems. Trends in Biosciences, 2014, 7(14), 1635-1637.

[25] Haque, M.M.; Pervin, E. Responses of genotypes and guti urea on yield and yield contributing character of transplant aman rice varieties (*Oryza sativa* L.). Scientia Agriculturae, 2015, 9(3), 172-179.

Biological properties of African swine fever virus Odintsovo 02/14 isolate and its genome analysis

Elsukova A.A.[1], Shevchenko I.V.[2], Varentsova A.A.[3], Puzankova O.S.[4], Zhukov I.Y.[5], Pershin A.S.[6], Remyga S.G.[7], Zinyakov N.G.[8],Mazloum A.[9], Vlasov I.N.[10], Igolkin A.S.[11], Lozovoy D.A.[12], Gruzdev K.N.[13], Vlasova N.N.[14]

[1-9,11-14]FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), 600901 Yur'evets, Vladimir, Russia.
[10]Rosgenediagnostics, 141400, Rabochaya str., house 2A apt. 1, Himki (town), Moscow region, Russia.

Abstract— *We performed analysis of the biological properties of African swine fever virus (ASF) isolate Odintsovo 02/14. Domestic pigs were inoculated with 50 (low) or 5000 (high) hemadsorbing doses (HAD) of the virus via intranasal (IN) or intramuscular (IM) routes, to investigate the pathogenesis of ASF virus Odintsovo 02/14 isolate. Our results indicated that filtered 10% spleen suspension of ASFV isolate Odintsovo 02/14 induced an acute disease in pigs, resulting in 100% mortality rate. For cultural viral suspension (3rd passage), produced in a PBM cells mortality rate was 85.7%.*

We also present an analysis of the complete genome of African swine fever virus (ASF) Odintsovo 02/14 isolate. It is 189 333 nucleotide long and contains more than 160 open reading frames (ORFs). Complete nucleotide sequence of the genome of Odintsovo 02/14 isolate was obtained using pyrosequencing method and used to determine differences between the nucleotide sequences in the genomes of Odintsovo 02/14 and Georgia 01/2007. The genome of ASF virus Odintsovo 02/14 contains substitutions, insertions and deletions in genes encoding structural, membrane, and regulatory proteins, DNA reparation enzymes, host immune response evasion proteins, and MGF genes.

The intergenic region I73R/I329L of Odintsovo 02/14 isolate contains 10-nucleotide long tandem repeat sequence, missing in Georgia 01/2007.

Keywords— African swine fever (ASF), experimental challenge, complete genome sequencing, intergenic region, tandem repeats.

I. INTRODUCTION

In 2007 ASFV was detected in the Caucasus region, first in Georgia and subsequently in Armenia, Azerbaijan, and many regions of Russia, including regions that border other countries in Europe and Asia. Epizootiological, pathogenic and immunogenic characteristics of the virus, as well as lack of an effective vaccine make ASFV a global threat to pig industry. According to the sequencing of PCR products and the hemadsorption inhibition reaction, this pathogen has been assigned to II genotype and seroimmunotype VIII [3, 33].

Russian isolates of ASFV induce an acute form of the disease, characterized by hemorrhagic syndrome and multisystem damage, high level of viremia and 100% mortality in domestic pigs.

The unique morphology of ASFV causes its high persistence and longevity in environment.

The causative agent of ASF is a large DNA-containing enveloped virus with a virion ~200 nm in diameter. [13]. ASFV virus belongings to the *Asfarviridae* family. Its particles consist of a nucleoprotein core ~80 nm in diameter. The genome containing electron-dense nucleoid enclosed in a lipid membrane and covered by an icosahedral capsid, made of 1892-2172 capsomeres. The virus acquires an external envelope during the budding process, thus forming extracellular virions about ~200 nm in diameter [2, 19].

Viral dsDNA is 170-193 kbp long, and it contains 150-167 ORFs. Generally, changes in the genome length are the result of variation in the number of tandem repeats within a certain gene or intergenic region [1, 27].

ASFV isolate Georgia 2007/1 has a genome of 189 344 bp and contains 166 ORFs. Chapman D. et al. demonstrated the maximum homology of Georgia 2007/1 isolate and Mkuzi 1979 isolate by phylogenetic analysis of the nucleotide sequences of 125 ORFs. In addition, they revealed some differences in the grouping of genes, which may be a result of recombination occurring during the evolution of virus [9].

High variability in groups of genes responsible for the virulence expression and interaction with the host requires constant monitoring of changes in a viral genome [19]. Comparison of the genomes of ASFV isolates of varying virulence can help in determining virulence responsible genes and develop a strategy for making an effective vaccine.

The first complete genome sequence for ASFV was obtained by Yanez et al. in 1995 for Ba71V strain, adapted to Vero cells [31]. Recently, sequences from strains of different genotypes became available for comparative analyses [9, 10, 12].

Currently, next generation sequencing (NGS) is the best method for viral genome studying. A single NGS procedure allows determining the entire nucleotide sequence even for large viruses, like Poxviruses, ASF, Herpes, etc. [24]. It is a key tool for detection of variable and marker regions in a genome, which are required for epidemiological studies and analysis of the distribution of viral subpopulations [30]. It also allows sequencing of nucleotides at the ends of the viral genome, which cannot be determined using methods based on PCR products sequencing analysis [32].

Comparison of genomic changes and changes in the biological properties of the ASFV will allow us to find out sources of the genome variability; to determine the hallmarks for differentiating isolates by pathogenicity; to track the evolution stages of the ASFV and to identify possible pathogen introduction sources.

Therefore, the purpose of this study was analysis of the biological properties of Odintsovo 02/14 isolate and its complete genome sequencing.

II. MATERIALS AND METHODS

2.1 Virus and cells

Virus was isolated from the spleen of a wild boar, shot in Tarakanovskiy forestry, Odintsovo district, Moscow region. Primary porcine alveolar macrophages (PAM) cell-culture [18] was used for the isolation of virus from the original field samples (10% filtered spleen suspension). Later, Odintsovo 02/14 virus was replicated in porcine bone marrow cells (PBM) for three consecutive passages [11, 18].

Cell cultures were infected with 0.1 HAD per cell. Examination of cultural properties was performed in primary porcine alveolar macrophages (PAM) and primary porcine kidney (PK). Alveolar macrophages from the lungs of healthy outbreed pigs were obtained by broncho-alveolar lavage with phosphate-buffered saline (PBS).

Two or three days old sub confluent monolayer cells were infected with the virus. First, cells were washed twice with maintenance medium MEM (Minimum Essential Media) containing 1% antibiotics and anti-fungal, then inoculated with the filtered samples. After adsorption, (60 min at 37°C in 5% CO_2), DMEM (Dulbecco's Modified Eagle's Medium) supplied with 10% FBS (Fetal Bovine Serum) was added to cultural mattresses. The cell cultures were observed for the presence of hemadsorption.

2.2 Virus titrations

The titer of ASF virus was determined by hemadsorption in PBM. PBM cells were added to 96-well microtiter plates. Cells were inoculated at dilutions of 1:10. Growth medium with 0.1% washed homologous red blood cells (RBCs) was added to each well. 10 folds diluted samples were added in quadruplicate to the plates and incubated for 6 days. The virus titer was calculated by the appearance of 50% of cells expressing hemadsorption as HAD_{50}/cm^3.

2.3 Hemadsorption types and its analysis

After three days of incubating the subconfluent monolayer of PBM, it was infected with a 0.1 HAD_{50}/cell and incubated for 48-72 hours in order to detect hemadsorption. Then the culture was photographed (15-20 images per sample) using microscope magnification of 400x. For every infected cell we counted the number of RBC's attached to it. 100-200 cells were counted in the field of view disposed along the diagonals of the sample. To avoid errors in the assessment of the hemadsorption type caused by the variations of the properties of cell cultures, the experiment was repeated three times on different batches of PBM cells. Statistical analysis included arithmetic mean values, standard error of arithmetic, evaluation of the reliability of the mean values, and significance of differences were performed using standard programs [28].

2.4 Animals

For experimental challenge with ASFV Odintsovo 02/14 isolates, we used 22 seronegative large white pigs; 8 in the first experiment and 14 in the second experiment , weighing 20-25 kg, aged 45-60 days, obtained from herds free of viral agents

in Vladimir region of Russia. Pigs were dewormed prior to arrival to our facility, and kept within the housing facilities for 7 days prior to the onset of the experiment for accommodation to the new environment.

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of FGBI ARRIAH, and conducted in compliance with local and federal guidelines. For this particular study, we used the lowest numbers of pigs previously shown to permit detection of statistical significances among treatments considering welfare principles. This minimum number of pigs required per treatment group was verified by the statistician before starting the experiments.

2.5 Direct inoculation studies

To study the biological properties of ASF virus Odintsovo 02/14 isolate and analyze the homogeneity of its population, we performed three experiments.

In the first experiment, we used 8 pigs. 6 pigs were assigned to 3 groups (two pigs in each group) and infected with 10% boar spleen suspension. In order to compare routes of inoculation group 1 was infected via intramuscular route (IM) [5×10^3 HAD_{50}], groups 2 and 3 via intranasal (IN) [5×10 HAD_{50} and $5\times10^3HAD_{50}$.] Two pigs were used for direct contact.

In the second experiment, we used virus from third passage PBM cell culture to infect the pigs. We used 14 animals, divided into 2 groups; in a first group 5 pigs were infected with 10 HAD_{50} through IM route, and two pigs were used for direct contact. In the second group, 5 pigs were infected by 50 HAD_{50} through IM route and 2 pigs were also used for direct contact.

In the third experiment, surviving animals from the previous experiment were infected with highly pathogenic ASFV isolate Boguchary 06/13 [10^3 HAD_{50}].

IM infection was carried out by introducing 2 cm^3 of virus-containing material in the right cucullaris muscle. Pigs assigned to IN inoculation were placed in sternal recumbency and inoculated with 2 cm^3 of virus suspension (1 ml per nostril). After inoculation of pigs we conducted daily temperature registration and observations. We investigated the level of viremia, virus presence in nasal and fecal swabs and duration of illness prior to animal death. Determination of the level of viremia in infected pigs was evaluated by DIF and PCR.

2.6 Direct contact (DC) transmission studies

This experiment was performed to characterize ASFV transmission from inoculated pigs and viral dynamics in pigs challenged by direct contact. Healthy contact pigs were initially housed in separate rooms. Infected pigs were inoculated using the intranasal (IN) method as described. Upon detection of pyrexia (rectal temperature $\geq40^{\circ}C$) in infected animals (5-6 DPI), contact pigs were transferred and housed with infected pigs.

2.7 Clinical evaluation and sample collection

The clinical assessment and rectal temperature monitoring were conducted daily. Sample collection (whole blood with EDTA, clotted blood for serum collection and swab samples from the nasal cavity and anus) were performed every two days. Blood were collected from the cranial cava vein. Nasal and anal swabs were collected using sterile cotton swabs. Following collection, swabs were immediately immersed in 1 ml of DMEM containing 5% antibiotics and antifungals; then all samples were stored in microtubes at −70°C until they were analyzed by virus isolation (VI) or PCR. Autopsy was performed on pigs as soon as possible following euthanasia or natural death. Tissues collected included: lung, thymus, liver, spleen, kidneys, and lymph nodes including: retropharyngeal, submandibular, gastrohepatic, renal and inguinal. Urine was also collected by direct aspiration from the urinary bladder at the time of necropsy. Collected tissue samples were placed in microtubes and stored at $-70^{\circ}C$.

2.8 Direct Immunofluorescence reaction

For the direct immunofluorescence (DIF) anti-p72 monoclonal antibodies – FITC (Ingenasa, Spain) was used. Fluorescence observations were performed using Olympus fluorescence microscope (Japan) at 400x magnification.

2.9 PCR

Viral DNA was extracted directly from suspensions of clinical samples using the DNA-Sorb DNA Purification Kit "Interlabservice" (Russia) following the manufacturer's protocol. Real-time polymerase chain reactions (RT-PCRs) were performed using the "ASF detection Kit" ("Interlabservice") as recommended by the manufacturer.

2.10 Virus and DNA purification

Extracellular virus was purified by sucrose gradient ultracentrifugation as described by Black D.N. and Brown F. (1976) [5]. ASF virus's DNA was extracted using the phenol/chloroform method [29].

For the pyrosequencing we needed to extract DNA from ASF virus that was replicated and harvested in PAM cells. In brief, the virus-containing suspension was centrifuged at 3000 rpm for 40 min at 4°C. The clarified virus-containing suspension was introduced into a hermetically sealable centrifuge tubes and centrifuged for 1 hour at 25.000 rpm. The viral pellets were resuspended in a small volume (no more than 10 cm^3) of PBS (pH 7.2-7.4). DNase I and RNase A (final concentration 50µg/cm^3) were added to the virus-containing suspension and this mixture was incubated for 0.5 hours at 37°C. Then pronase E was added to a final concentration of 200µg/cm^3 and incubated for 6-12 hours at 37°C.

Further, virus-containing suspension was applied to a sucrose step gradient 25%-35%-50% and centrifuged at 25,000 rpm for 1, 5 hours. Virus band located over 50% sucrose cushion was collected in a minimal volume (1-2 ml) and used to extract the viral DNA, sample was diluted 3-4 times with 1 × TE buffer. Viral DNA was extracted using the phenol-chloroform method by Enjuanes L. et al. [16].

Analysis of the sample purity and determining its concentration was performed spectrophotometrically, by measuring optical density (A) at wavelengths of 260 and 280. Integrity of DNA was checked by electrophoretic separation on an agarose gel.

2.11 Pyrosequencing of ASFV genome

To determine the complete nucleotide sequence of ASF virus we used pyrophosphate sequencing (454 Life Sciences) on automated sequencer GS Junior (Roche, Germany) according to the manufacturer's protocols. The fragmentation of genomic dsDNA was performed by nebulization with nitrogen using «GS Rapid Library Prep Nebulizers», «GS RL Buffers Kit» (Roche, Germany) kits following the manufacturer's instructions. Further complex molecular genetics operations for the preparation of the library and sequencing was performed using the following kits: «GS Rapid Library Rgt/Adaptors Kit», «GS Rapid Library MID Adaptors Kit», «GS Junior emPCR Reagents (Lib-L)», «GS Junior emPCR Bead Recovery Reagents», «GS Junior emPCR Oil & Breaking Kit», «GS Junior Seq. Reagents and Enzymes», «GS Junior Sequencing Buffers», «GS Junior Packing Beads & Supplement CB» (Roche, Germany) according to the manufacturer's instructions. For genome assembling we used GS De Novo Assembler and GS Reference Mapper (454 Life Sciences) software.

2.12 Comparative analysis

Detection of changes in viral genes was performed by comparative analysis of the full-genome sequence of Odintsovo 02/14 isolate and full-genome sequence of Georgia 2007/01 isolate (GenBank). Alignment of ASF virus genome sequences was performed using the ClustalW (http://www.clustal.org/clustal2/) method.

2.13 Statistical analysis

In this study we used STATGRAPHICS® Centurion XV, version 1.15.02 [28] for data processing. The statistical analysis was carried out using method of analysis of repeated variations [8].

2.14 ELISA

To detect the presence of specific antibodies in the serum of pigs ELISA was carried out using Ingenasa commercial kit (Spain) according to the manufacturer's instructions.

2.15 DNA electrophoresis

DNA fragmentation was evaluated by electrophoretic separation in 0.7% agarose gel.

III. RESULTS

3.1 Cultural properties.

To evaluate properties of Odintsovo 02/14 isolate in PAM culture, we chose the following parameters: hemabsorption presence and type, time and titer of viral accumulation (Table 1).

TABLE 1
CULTURAL PROPERTIES OF ASF VIRUS ODINTSOVO 02/14 ISOLATE ON PAM

Isolate	Passage	Number of RBC attached to the infected cell	Virus accumulation titer (lg HAD_{50}/cm^3)	Virus accumulation time (days)
Odintsovo 02/14	1	>50	5,21 ± 0,36	7
	2	30-50	6,66 ± 0,14	6
	3	10-30	7,02 ± 0,12	5

The titer of the third passage increased from 5.21 to 7.02 lg HAD_{50}/cm^3, while the accumulation time decreased from 7 to 5 days.

Furthermore, throughout the course of serial passaging we detected changes in hemadsorption type. In the first passage, 50% of infected cells were adherent to 30-50 erythrocytes, 25% of infected cells adhered to less than 30 erythrocytes, and 25% of infected cells to more than 50 erythrocytes, while in the third passage, 50% of infected cells adhered to less than 30 erythrocytes, 35% of infected cells were adherent to 30-50 erythrocytes and 15% had adhesion with 50 erythrocytes (Figure 1).

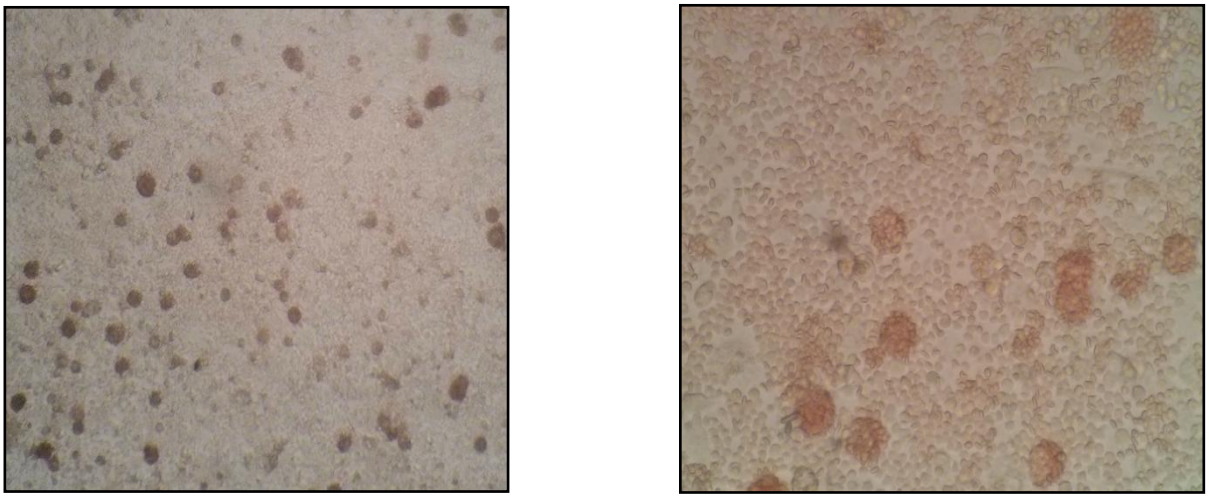

FIGURE 1 - HEMADSORPTION IN PAM CELL CULTURE INFECTED WITH ASF VIRUS ODINTSOVO 02/14 ISOLATE (left – magnification at 200x, right - magnification at 400x).

In primary cell culture PK, reproduction properties of Odintsovo 02/14 isolate were analyzed using 10% filtered spleen suspension (5,21 ± 0,36 lg HAD_{50}/cm^3). Cell cultures were infected with 0.1 HAD per cell (Figure 2).

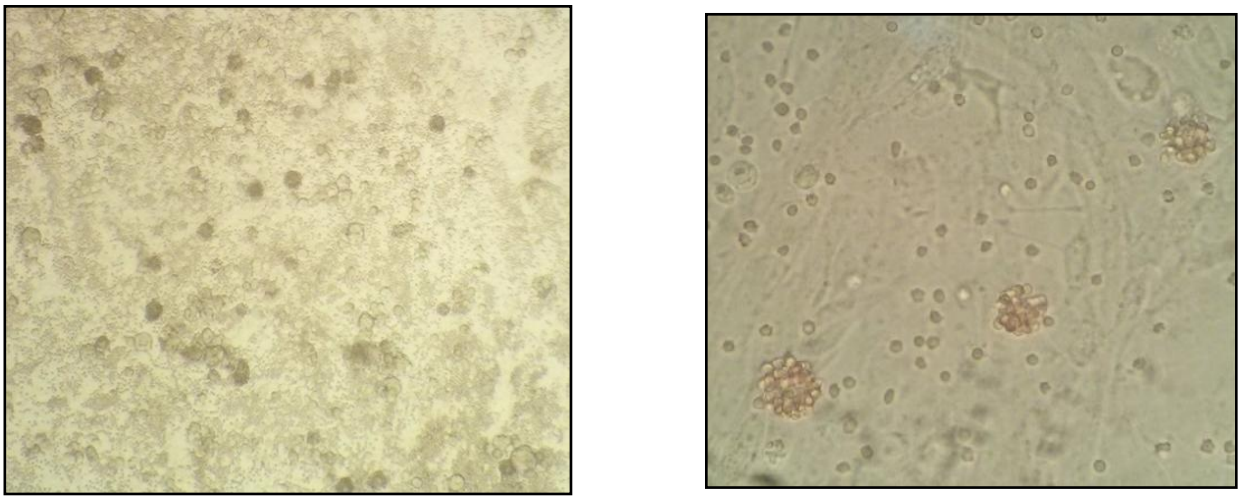

FIGURE 2 - CPE AND HEMADSORPTION BY ASF VIRUS ODINTSOVO 02/14 ISOLATE IN PK PRIMARY CELL CULTURE (left - magnification 200x, right - magnification 400x).

Five serial passages in PK primary cell culture were performed; the passage duration was 7 days (results are given in Table 2).

TABLE 2
STUDY OF ODINTSOVO 02/14 ISOLATE REPRODUCTION IN PK PRIMARY CELL CULTURE

Isolate	Passage	Number of RBC attached to the infected cell	Virus accumulation titer (lg HAD_{50}/cm^3)	Virus accumulation time (days)
Odintsovo 02/14	1	<50	5,08±0,45	9
	2	<50	5,46±0,31	8-9
	3	10-30	6,09±0,41	6-7
	4	10-30	6,36±0,12	6
	5	10-30	6,65±0,12	6

During the experiment (Table 2) Odintsovo 02/14 isolate was found to be able to reproduce in PK primary cell culture. Replication began without prior adaptation, and hemadsorption was detected at the first passage. By the fifth passage the accumulation time decreased from 9 to 6 days, while the accumulation titer increased from 5.08 to 6,65lg HAD_{50}/cm^3.

3.2 Biological properties

3.2.1 First experiment

First group of pigs (5000 HAD/IM) showed hyperthermia with maximum temperature level 41,5°C at 6 days post-infection (d.p.i.). Animals died on13 d.p.i.

Within the second groud (5000 HAD/IN) only one pig showed hyperthermia on 6 d.p.i and died on 14 d.p.i. The other pig from this group developed a stable hyperthermia with maximum temperature 41,8° C only on 16 d.p.i., and died on 22 d.p.i.

Within the third group of two pigs (50 HAD/IN) the onset of hyperthermia was observed on 24-25 d.p.i. These animals were slaughtered on 30-31 d.p.i. Maximum temperature reached 41,8°C.

Regarding direct-contact pigs, the maximum temperature reached was 41,6°C. Clinical signs were observed at 26 d.p.i., and death of animals occurred at 32-33 d.p.i. (Figure 3).

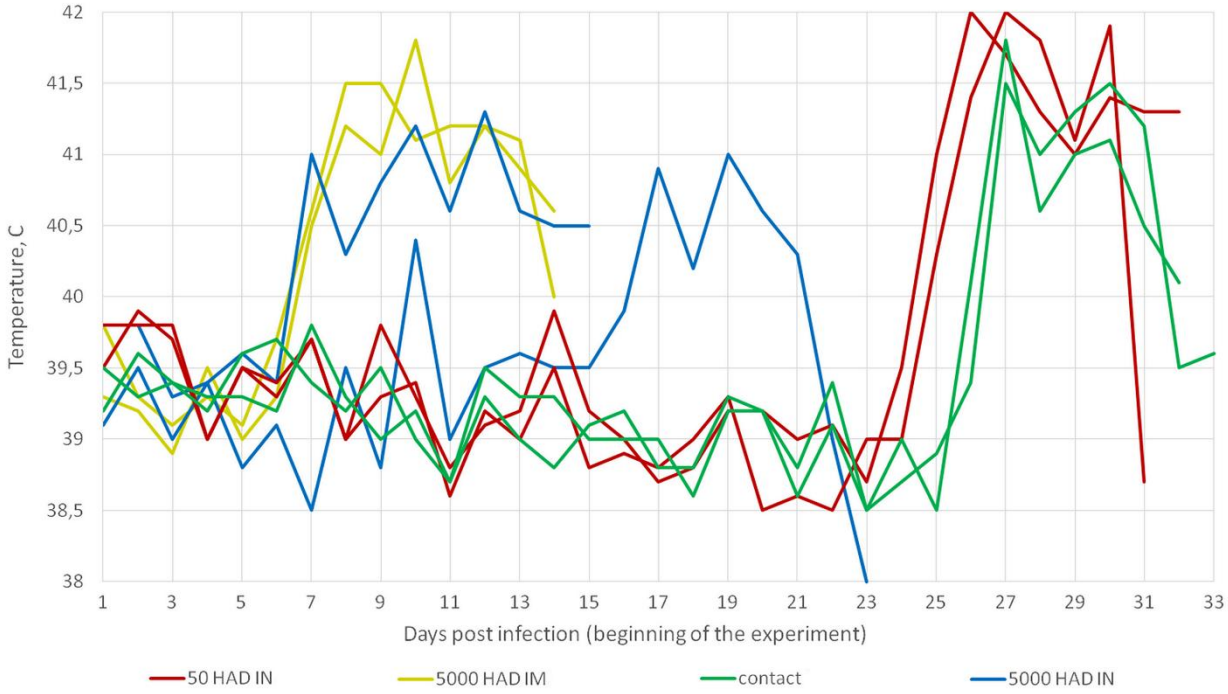

FIGURE 3 – TEMPERATURE GRAPH OF PIGS INFECTED WITH ASFV ODINTSOVO 02/14 ISOLATE, (FIRST EXPERIMENT).

At autopsy, all dead animals demonstrated changes, specific to ASF: splenomegaly, hyperplasia and hyperemia of the regional lymph nodes, hemorrhages under the renal capsule, and in the bladder lining. These changes were less expressed in animals that died in the early stages (14 d.p.i.), and were more expressed in animals that died during the later stages (31-33 d.p.i.).

Blood and internal organs samples of all animals were investigated by HAD reaction (Table 3), DIF and PCR, for the presence of ASF virus, its antigens and genome, respectively.

TABLE 3
VIREMIA LEVEL ANALYSIS IN PIGS, INFECTED BY ASF VIRUS ODINTSOVO 02/14.

Group	Dose (lg HAD_{50} cm^3)*	Days post infection														
		3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
1	5000 HAD IN	1,77± 0,23	2,51± 0,32	5,07± 0,11	6,51± 0,11	7,45± 0,18	6,08± 0,17	4,53± 0,33	7,21± 0,45	6,46± 0,35	5,87± 0,13					
		1,68± 0,12	3,53± 0,13	6,54± 0,19	7,03± 0,11	7,66± 0,14	7,05± 0,22									
2	50 HAD IN	-	-	-	-	-	-	< 1,0	2,34± 0,32	3,43± 0,19	5,07± 0,12	6,08± 0,27	6,66± 0,13	7,45± 0,18	6,74± 0,29	6,07± 0,12
		-	-	-	-	-	-	1,87± 0,13	2,54± 0,11	3,53± 0,11	5,56± 0,15	7,05± 0,22	4,86± 0,24	7,66± 0,14	6,16± 0,40	
3	5000 HAD IM	< 1,0	2,68± 0,14	4,37± 0,21	6,66± 0,13	7,02± 0,51	6,45± 0,18									
		< 1,0	2,56± 0,07	4,29± 0,61	6,57± 0,18	7,56± 0,41	6,66± 0,14									
4	Contact			-	-	-	-	-	-	-	1,77± 0,23	3,53± 0,13	5,43± 0,17	6,96± 0,17	7,45± 0,18	6,07± 0,12
				-	-	-	-	-	-	-	1,68± 0,11	2,28± 0,14	3,53± 0,11	6,04± 0,42	7,66± 0,14	5,07± 0,11

Therefore, the mortality rate for pigs in a first experiment was 100%.

3.2.2 Second experiment

In a second experimental challenge we used Odintsovo 02/14 isolate (3rd passage in PBM cell culture) to test the structure of the viral population and to determine the biological properties of this isolate. Additionally, we reduced the infection dose and used 14 animals.

Since we determined differences in the duration of the disease, it was necessary to find out correlations between changes of the biological properties and genome of Odintsovo 02/14 isolate.

In the second experiment 4 out of 5 pigs infected with a 50 HAD dose died betweeen 10-16 d.p.i.;. 4 out of 5 pigs infected with 10 HAD dose died between 10-18 d.p.i.. There were surviving pigs in every group. Pig №3 infected with a 10 HAD dose showed no reaction to the introduction of the virus. Pig №8 which was infected by 50 HAD dose expressed single temperature peak at 25 d.p.i. (Figure 4).

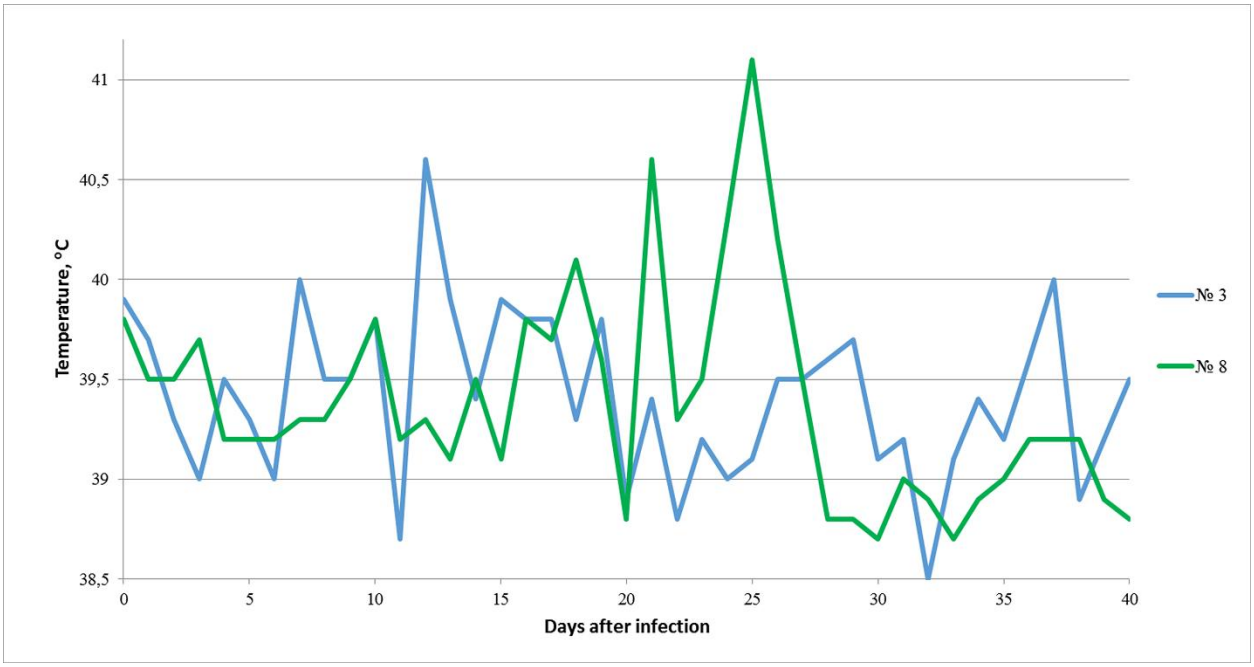

FIGURE 4 – TEMPERATURE GRAPH OF SURVIVING PIGS, INFECTED WITH ASFV ODINTSOVO 02/14 ISOLATE FROM 3RD PASSAGE IN PBM CELL (SECOND EXPERIMENT).

We did not register any other specific reactions to ASFV injection in pigs for 40 d.p.i. (observation period).

Analysis of the antibodies levels in surviving pigs serum showed that in pig №3 antibodies were detected at 9 d.p.i., while for pig №8 antibodies were detected at 12 d.p.i. (Table 4).

3.2.3 Third experiment

At 40 d.p.i. we challenged both surviving pigs by 1000 HAD dose of high virulent ASF virus Boguchary 06/13 isolate, described previously [25]. At the observation time (90 days) we did not registered any symptoms of ASF in these pigs. Therefore, these pigs acquired protective immunity against ASF.

3.3 Genome analysis

We prepared three series of ASFV Odintsovo 02/14 - containing culture fluid with a total volume of more than 7 liters for virus purification and DNA isolation (Table 4).

TABLE 4
VIRUS PURIFICATION AND ASF VIRUS DNA EXTRACTION RESULTS

№	Volume ($см^3$)	Virus titer ($lg\ HAD_{50}/cm^3$)	Purity of the isolated DNA (A_{260}/A_{280})	Resulting DNA concentration ($\mu g/cm^3$)
1	2500	6,02	~2,03	64,2
2	2400	5,67	~1,89	72,1
3	2300	4,75	~1,93	39,6

As seen from the Table 4, we obtained unfragmented genomic ASF virus DNA. Sample 1 had optimal indicator of purity and concentration, therefore it was chosen for further pyrosequencing.

3.4 Comparative analysis.

The genome of Georgia 2007/01 isolate is 189 344bp long (GenBank accession no.: FR682468.) However, sequence analysis of Odintsovo 02/14 isolate revealed a genome of 189 333bp long (accession no. KM262844). The majority of differences were located in the left (LVR) and right variable (RVR) regions of the genome, with 45.8% of them localized in the LVR.

The comparative analysis indicated that the genome of ASF virus Odintsovo 02/14 isolate has 22 mononucleotide insertions, 3 polynucleotide insertions, including a direct tandem repeat, 35 mononucleotide and 5 polynucleotide deletions, also 7

substitutions. In comparison to Georgia 2007/01 genome (189 344 nucleotides), these changes in the Odintsovo 02/14 genome constitute to ~ 0.038% of its total length (Table 5).

The first key difference was the presence of the tandem repeat sequence GGAATATATA in the intergenic region between genes I73R and I329L of Odintsovo 02/14 isolate, which was absent in Georgia 2007/1 genome.

TABLE 5
CHANGED GENES OF ODINTSOVO 02/14 ISOLATE

ORF	Location	Gene	Function	Change
4	4 114	L83L	unknown (BA71V-L83L)	+ T
7	5 670	ASFV_G_ACD_00070	ASFV-Georgia_Final_2-007	+ A
8	6 095	MGF_110-1L	110 Multigene	C/T
12	7 940	ASFV_G_ACD_00120	ASFV-Georgia_final-012	+ A
29	16 663	ASFV_G_ACD_00290	ASFV-Georgia_final-029	- GG
54	43 599	MGF_505-9R	505 Multigene	A/G
117	124 803	CP204L	p30 phosphoprotein. Involved in virus entry.	+ T
124	133 521	NP419L	DNA ligase	T/C
147	161 252	QP383R	NifS like protein (BA71V-QP383R)	- A
162-163	172 407	I243L-I73R	New intergene region	+ GAATATATAG
168	175 376	I196L	unknown (BA71V-I196L)	T/C

According to the analysis of changes and summarizing the data presented in Table 5, we can conclude, that the genomic structure of ASF virus Odintsovo 02/14 differs from Georgia 2007/1 isolate. Changes include: genes encoding structural and functional proteins of the virus, replacements of genes in multigene families (MGF_110-1L, MGF_505-9R) which are responsible for virulence [15]. Mapping of the changes in ASFV to its functional map indicated that some of the changes occurred in genes of multigene families, pA403, pKP360 and pL356L, which according to L. Dixon can lead to reduction of virulence of the ASF virus.

IV. DISCUSSION

Our study has been conducted at the Reference Laboratory for ASF (FGBI "ARRIAH"), attempting to resolve the intra-genotypic relationships of ASF viruses that have been causing outbreaks in Russian Federation over a period of 8 years, and to detect epidemiological links that may exist between outbreaks from different regions. Nevertheless, the low levels of intra-genotype diversity among field isolates collected for the past three years make it harder to clarify the relation within genotype.

Belyanin A.S. et al noted that, for Russian Federation ASFV isolates 2007-2012 the incubation period was 4.0 (3.0-5.0) days and the infectious period (duration of illness) - 6.5 (5.0-8.0) days on experimental swine. There were edema and multiple hemorrhages in all organs of infected animals with viremia level reaching HAD 6,5-7,5 lg_{50}/cm^3 with 100% mortality rate [3].

We analyzed the influence of infection routes on illness duration and death onset on pigs challenged in the same dose (5000 HAD). The IN challenge led to prolongation of manifestation of the disease for 2-10 days and postponing of pigs death for 1-9 days comparing to IM infection.

The influence of the infecting dose on development of the disease was noted in our earlier studies. In this study (*Experiment 1*) clinical signs in pigs infected with 5000 HAD occurred 8-18 days earlier compared to pigs infected with 50 HAD. Effects of dose and infection route in terms of the onset of hyperthermia and death were confirmed in similar research in the study of the biological properties of other ASF virus samples, isolated in Russian Federation.

We noticed an increase in the duration of the incubation period. In groups 1 and 2, the incubation period for the two pigs infected IM and one infected IN with 5000 HAD lasted for 6 days, which converges with the data obtained from previously described isolates. However, for one pig infected IN with 5000 HAD the incubation period lasted for 16 d.p.i..

In group 3 the incubation period for two pigs infected IN with 50 HAD lasted 24-25 d.p.i.. The longest incubation period (~28 days) was observed in a contact group. The average life span post infection for contact animals was ~7 days as therefore did not differ from other groups.

Thus, the duration of the incubation period was significantly longer than previously recorded for Russian isolates 2007-2011 (2-4 d.p.i.) [3, 4, 7].

S. Blum et al. conducted a study using high, moderate and low infection doses of ASFV Caucasian strains to assess the risk of development of chronic disease and virus infection. They showed that low dose infection may lead to prolonged incubation periods and clinical courses, and that 1000 HAD are not sufficient to produce disease in healthy wild boars [6, 7].

Differences in the incubating period span for pigs, challenged with 50 HAD dose could be due to an individual differences in the immune response of pigs or heterogeneous composition of Odintsovo 02/14 isolate population.

To analyze population structure of Odintsovo 02/14 isolate we used two samples: one from the spleen of a wild boar, and second was passed no more than 3 times in PBM cells, as according to P.W. Krug no considerable changes in the viral genome can occur in these circumstances if the virus population is homogeneous [20]. Otherwise, if it is heterogeneous, reproduction in the culture cells can change the structure of the population. The fact that 2 pigs withstanded the challenge with 3rd passage of Odintsovo 02/14 isolate in PMB cell culture without any simptoms may indicate the presence of an avirulent component in the viral population.

Mapping of the changes in ASFV to its functional map by L.K. Dixon et al., indicated changes in the genes of a multigene family MGF_505, as well as genes encoding CD2v protein and both DNApol, and Guanylyl transferase enzymes, which may affect the rate of the virus replication, and therefore virulence [14].

In this study, the detection of gene(s) responsible for changes in the biological properties of Odintsovo 02/14 isolate was not possible. However it allowed us to make assumptions about the causes of increased incubation period through the analysis of the correlation between biological properties and alterations in ASFV genome.

In the genome of Odintsovo 02/14 we found 22 mononucleotide insertions, 3 polynucleotide insertions, including a direct tandem repeat GAATATATAG, 35 mononucleotide and 5 polynucleotide deletions, and 7 substitutions.

The 454 GS Junior sequencer (Roche) may produce mistakes sequencing homopolymer sequences longer than 6 nucleotides; it permits 0.38 errors per 100 bases. In general, those mistakes are mononucleotide deletions which can be formed even at a high depth of coverage [22]. Analysis of the Odintsovo 02/14 nucleotide sequence allowed us to identify genes containing incontestable changes - 37.5% of the variation of the total amount.

These included changes (T/C) in NP419L gene, encoding an ATP-dependent viral DNA-ligase which is part of the viral DNA-repair enzymes complex. It is characterized by extremely high tolerance for errors at the crosslinking chains reparation [34].

We also detected changes in the nucleotide sequences of ASFV_G_ACD_00070, I196L and M448R genes, those which have an unknown function yet, and genes of multigene families MGF_360-1L, MGF_360-2L, MGF_110-1L, MGF_505-9R. It is possible that these changes have led to an increased incubation period in pigs infected with Odintsovo 02/14.

Changes in the intergenic areas of the genome may also have an impact on virulence, for example by affecting promoter or other regulatory sequences [26]. We found out that the genome of ASFVOdintsovo 02/14 isolate has a tandem repeat GGAATATATA in the nucleotide sequence of the intergenic region I73R/I329L. Its presence according to Gallardo C. et al., was not identified in Russian isolates between 2007 and 2010. This tandem repeat was noted by the authors only in European isolates 2013-2014 years and in the genome of Ukrainian isolate in 2012 [17]

V. CONCLUSION

Cultural properties of Odintsovo 02/14 isolates are significantly different amongst other Russian isolates 2007-2014. It manifests a decreased amount of RBC's, attached to an infected cell, which according to L.Dixon is related to attenuation of the virus.

Mortality in infected animals reached 100% for the samples of spleen suspension and 85.7% for the 3rd PBM cells passage samples, when we used 50 HAD dose for the challenge, which may indicate the heterogeneity of the viral population of this isolate and presence of viral variants with different pathogenicity.

A comparative analysis of the nucleotide sequences of Odintsovo 02/14 and Georgia 2007/1 ASFV isolates identified differences in genes responsible for virus attachment, cell penetration and host immune system evasion genes. Changes were found in nucleotide sequences of genes I196L, NP419L, ASFV_G_ACD_00070, M448R, MGF_360-1L, MGF_360-2L, MGF_110-1L, MGF_505-9R and GGAATATATA tandem repeat sequence in the intergenic region I73R/I329L, which is absent in the ASF isolates collected before 2012.

The analysis highlighted group of genes of Odintsovo 02/14 isolate, where changes are undoubted (37.5% of the total variations, where 23.6% are mononucleotide deletions/insertions). These include nucleotide replacement T/C in a NP419L gene encoding the ATP-dependent viral DNA-ligase complex, included in the viral reparation. It is characterized by an extremely high tolerance for errors in the presence of crosslinking chains by crosslinking nick breaks [15].

Due to variations in a number of tandem repeats in a nucleotide sequence of the ASFV I196L gene it was used for phylogenetic analysis of ASFV isolates [15]. These changes may have led to an increased incubation period in pigs infected with Odintsovo 02/14. The detected changes in the gene sequences require further study.

REFERENCES

[1] Afonso, C.L., Piccone, M.E., Zaffuto K.M., Neilan J.; Kutish G.F., Lu Z.; Balinsky C.A., Gibb T.R., Bean, T.J., Zsak, L.; Rock D.L. 2004. African swine fever virus multigene family 360 and 530 genes affect host interferon response // Journal Of Virology. 78(4): 1858-1864.

[2] Arias M., Sanchez-Vizcaino J.M., Morilla A. [et al.] 2002. African swine fever eradication: the Spanish model// Trends in Emerging Viral Infections of Swine / ed. A. Morilla, K. Jin, J. Zimmerman. –1st ed. – Ames, Iowa, USA: 133-139.

[3] Balyshev V.M., Kurinnov V.V., Tsybanov S.Zh., Kalantaenko Yu.F., Kolbasov D.V., Pronin V.V., Korneva G.V. 2010. Biological properties of African swine fever virus isolated in the Russian Federation.// Veterinariya. 7: 25-27.

[4] Belyanin S.A., Vasilev A.P., Kolbasov D.V., Tsybanov S.Zh., Balyshev V.M., Kurinnov V.V., Chernykh O.Yu. 2011. Virulence of African swine fever isolates // Veterinary of Cuban. 5: 9-11.

[5] Black, D.N.; Brown, F. 1976. Purification and physicochemical characteristics of african swine fever virus.// Journal Of General Virology. 32(3): 509-518.

[6] Blome S., Gabriel C., Beer M. 2013. Pathogenesis of African swine fever in domestic pigs and European wild boar.// Virus Research 173: 122– 130.

[7] Blome, S., Gabriel, C., Dietze, K., Breithaupt, A., Beer, M., 2012. High virulence of African swine fever virus caucasus isolate in European wild boars of all ages.// Emerging Infectious Diseases 18 (4): 708.

[8] Box, G.E.P.; Hunter, W.G.; Hunter, J.S. 2005.Statistics for Experimenters: Design, innovation and discovery. (eds). Wiley. 2nd edition. 664 p.

[9] Chapman D.A.G., Darby A.C., Da Silva M., Upton C., Radford A.D.; Dixon L.K. 2011. Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus.// Emerging Infectious Diseases. 17(4): 599-605.

[10] Chapman, D.A.G.; Tcherepanov, V.; Upton, C.; Dixon, L.K. 2008. Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. Journal Of General Virology. 89(2): 397-408.

[11] de León P., Bustos M. J., Carrascosa A.L. 2012. Laboratory methods to study African swine fever virus.// Virus Research; 10: 173(1) DOI: 10.1016/j.virusres.2012.09.013

[12] de Villiersa, E.P.; Gallardo, C.; Arias, M.; da Silva, M.; Upton, C.; Martin, R; Bishop, R.P. 2010. Phylogenomic analysis of 11 complete African swine fever virus genome sequences.// Journal Of Virology. 400(1): 128–136.

[13] Dixon, L. K.; Costa, J. V.; Escribano, J. M.; Rock, D.L.; Vinuela, E.; Wilkinson P.J. 2000. Family Asfarviridae, p. 159-165. In M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.)// Virus taxonomy: seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.

[14] Dixon, L.K.; Abrams, C.C.; Bowick G.; Goatley, L.C.; Kay-Jackson, P.C.; Chapman, D.A.G.; Liverani, E.; Nix, R.; Silk, R.; Zhang, F. 2014. African swine fever virus proteins involved in evading host defence systems. // Veterinary Immunology and Immunopathology. 100(3-4): 117-134.

[15] Dixon, L.K.; Chapman, D.A.G.; Netherton, C.L.; Upton C. 2013. African swine fever virus replication and genomics.// Virus Research. 173(1) 3-14.

[16] Enjuanes L, Carrascosa AL, Viñuela E. Isolation and properties of the DNA of African swine fever (ASF) virus.// J Gen Virol.1976 Sep;32(3):479–492.

[17] Gallardo C. et al. 2014. Genetic Variation among African Swine Fever Genotype II Viruses, Eastern and Central Europe. //Emerging Infectious Diseases. 9: 1544-1547.

[18] Isolation of african swine fever virus (ASFV) in porcine macrophages and haemoadsortion test / Madrid, Spain: 2014. – 6 p.

[19] Kleiboeker S.B.; Kutish G.F.; Neilan J.G.; Lu Z., Zsak L., Rock D.L. 1998. A conserved African swine fever virus right variable region gene, I1 1L, is non-essential for growth in vitro and virulence in domestic swine.// Journal Of General Virology. 79(5): 1189-1195.

[20] Krug [et al.] P.W. The progressive adaptation of a Georgian isolate of African swine fever virus to Vero cells leads to a gradual attenuation of virulence in swine corresponding to major modifications of the viral genome // Journal of Virology. – 2015. – Vol. 89, № 4. – P. 2324-2332.

[21] Li, H.; Durbin, R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform.// Bioinformatics. 26: 589–595.

[22] Loman N. J., Misra R.V., Dallman T.J. (et al.) 2012. Performance comparison of benchtop high-throughput sequencing platforms.// J. Nature Biotehcnology. 30(5): 434-462.

[23] Malmquist W.A., Hay D. 1960. Hemadsorption and cytopathic effect produced by ASFV in swine bone marrow and buffy coat cultures.// Am. J. Vet. Res.; 21:104–108.

[24] Metzker M.L. 2010. Sequencing technologies - the next generation.// Nature Reviews Genetics. 11: 31-46.

[25] N.N. Vlasova, A. A. Varentsova, I. V. Shevchenko, I. Yu. Zhukov, S. G. Remyga, V. L. Gavrilova, O. S. Puzankova, A. A. Shevtsov, N. G. ZinyakovK. N. Gruzdev. Comparative Analysis of Clinical and Biological Characteristics of African Swine Fever Virus Isolates from 2013 Year Russian Federation // British Microbiology Research Journal, Vol.: 5, Issue.: 3 P. 203-215

[26] Portugal R., Coelho J., Hoper D., Little N. S., Smithson C., Upton C., Martins C., Leitao A. and Keil G. M. Related strains of African swine fever virus with different virulence: genome comparison and analysis// Journal of General Virology (2015), 96, pp.408–419, DOI 10.1099/vir.0.070508-0

[27] Sogo, J.M., Almendral, J.M., Talavera A., Vinuela E. 1984. Terminal and internal inverted repetitions in african swine fever virus DNA.// Journal Of Virology. 133: 271–275.

[28] STATGRAPHICS®Centurion XV User Manual. [Electronic resource] – Access mode. Available: http://gendocs.ru/v31715/программа_-_statgraphics_centurion_xv

[29] Genetic identification and nucleotide sequence of the DNA polymerase gene of African swine fever virus / A. Martins [et al.] // Nucleic Acids Research. – 1994. – Vol. 22, № 2. – P. 208–213.

[30] Torresi C. Application of Next-Generation Sequencing (NGS) technologies to the African Swine Fever Virus (ASFV) // Short Term Mission Report- www.sidilv.org/.../76-report-borse-di-studio-cla...

[31] Yanez, R.J.; Rodriguez, J.M.; Nogal, M.L.; Yuste, L.; Enriquez, C.; Rodriguez, J.F.; Vinuela, E. 1995. Analysis of the Complete Nucleotide Seqence of African Swine Fever Virus.// Journal Of Virology. 208(1): 249-278.

[32] Zhou X., Ren L., Li Y., Zhang M., Yu Y., Yu J. 2010. The next-generation sequencing technology: A technology review and future perspective.// Science China Life Sciences. 53: 44–57.

[33] Genotyping field strains of African swine fever virus by partial p72 gene characterisation / A. Bastos [et al.] // Archives of Virology. – 2003. – Vol. 148, № 4. – P. 693–706.

[34] Lamarche, B.J. ASFV DNA polymerase X is extremely error-prone under diverse assay conditions and within multiple DNA sequence contexts / B.J. Lamarche, S. Kumar, M.D. Tsai // Biochemistry. – 2006. – Vol. 45, № 49. – P. 14826-14833.

The Role of Cell Wall-Degrading Enzymes in the Development of Anthracnose Disease Caused by *Colletotrichumtruncatum* in the Chilli

Remli N.A.M.[1], Shah U.K.M[1,2*], Ariff A.B[1,2], MohdTermizi Yusof[3], Lim H.S[4]

[1]Department of Bioprocess Technology, Malaysia
[2]Bioprocess and Biomanufacturing Research Center, Malaysia
[3]Department of Microbiology, Faculty Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia,
[4]Agrobiodiversity and Environmental Research Centre, Malaysian Agricultural Research and Development Institute, 43400 Serdang, Selangor, Malaysia.
*Corresponding Author – Assoc. Prof. Dr. UmiKalsom Md Shah
Associate Professor in Bioprocess Technology, Department of Bioprocess Technology, Faculty Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Abstract— *The ability of Colletotrichumtruncatum CP2 in producing pectinolytic and cellulolytic enzymes was evaluated by shake flask fermentations. The results of enzymatic activity experiment indicated that PG was the first cell wall-degrading enzymes detected and the activities obtained were higher (0.24±0.10 U/mL) than other enzymes, which appeared later and in lower amount. After the cell wall was degraded by the action of PG, further degradation of the cell wall was affected by pectin methylesterases, pectin lyase, pectate lyase and cellulases. The disparity in enzymatic activity at different intervals may suggest their specific role for pathogenesis at proper timings.*

Keywords— Chilli anthracnose; Colletotrichumtruncatum, cell wall-degrading enzymes, pectinolytic, cellulolytic.

I. INTRODUCTION

Chilli (*Capsicum annuum L.*) belongs to the Solanaceae family is a profitable crop worldwide as it is not exclusively used as a spice in numerous cooking styles but also found to have numerous therapeutic properties. Chillies are commercially grown in tropical and sub-tropical countries like India, China, Japan, United States of America and African countries (Saxena *et al.*, 2016). Nonetheless, numerous limitations have significantly decreased chilli production worldwide. Plant disease caused by mainly fungi is the major constrain in chilli production worldwide as it can affect both tropical and subtropical region and even in temperate region.

The secretion of cell wall-degrading enzymes by fungal pathogen is an important factor that contributes to the pathogenicity of fungal pathogens during interaction with its host. These cell wall-degrading enzymes enable the fungal pathogens to penetrate into the cell wall more easily due to the decomposition of the cell wall polyshaccharides. Subsequently, the fungal pathogens will acquire the carbon from the decomposed plant tissues. Several studies have addressed the cell wall-degrading enzymes secretion by fungal pathogens and its specific relation with pathogenicity (Niture, 2008).

Recently, most research has been intensified on the pectinolytic enzymes secreted by fungal pathogens. The virulence of many pathogens has been suggested to be dependent on the level of pectinolytic enzymes produced during infection (Rogers *et al.*, 2000).However, the specific role of these enzymes in pathogenesis of fungal pathogen in chilli is still unknown. Till date, there are no published reports on pectinolytic enzymes activities related to pathogenicity of *C. truncatum*in chilli anthracnose. The aim of this study was to determine the production of cell wall-degrading enzymes by *C. truncatum* CP2 in submerged fermentation as a preliminary step to establish the role of these enzymes in the *C. truncatum* CP2 and chilli interaction.

II. MATERIALS AND METHODS

2.1 Microorganism

Colletotrichumtruncatum CP2 was used in this study. The fungal isolate was isolated from lesions of chilli fruit. The pure culture was transferred to PDA slants and maintained at 4 °C. *C. gloeosporiodes* was used as a reference (Heng *et al*., 2011).

2.2 Medium composition for enzyme production

In this study, basal medium was used as the medium for the production of cellulases and pectinases under submerged fermentation. Composition of basal medium used was as follow (g/L): (NH4)2SO4, 1.4; K_2HPO_4, 6.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.1 and carbon source (pectin or carboxymethyl cellulose), 10.

2.3 Production of cellulases and pectinases by *C. truncatum* CP2 in submerged fermentation

Enzyme production was carried out under submerged fermentation. The basal media (pH 5.0) were inoculated with 8 mm diameter culture discs of the fungal isolate. The cultures were harvested after incubation at 30°C for 2, 4, 6, 8, 10 and 12 days and centrifuged at 10,000 rpm for 20 min before determining the enzyme activity.

2.4 Pectinolytic enzymes assays

Polygalacturonase (PG) activity, the method of Nelson and Somogyi (Nelson, 1944) was used to measure the release of reducing groups from polygalacturonic acid. The reaction mixture contained 1.8 mL of 1% PGA in 50 mM sodium acetate buffer (pH 4.8) and 0.2 mL of crude enzyme. The boiled enzyme served as a control. The reaction mixture was then incubated at 40°C for 30 min followed by addition of 3 mL of 3, 5-dinitrosalicyclic (DNS) reagent. Reaction was stopped by heating the reaction mixture at 100 °C for 15 min. Then, the tube was cooled at room temperature before adding 1 mL of Rochelle salt. The absorbance was read at 545 nm using a spectrophotometer. The amount of reducing sugars was calculated using D-galacturonic acid as a standard. The amount of enzyme releasing 1 µmol of galacturonic acid per min at pH 4.8 and 40°C was considered as one enzyme unit.

Pectin methylesterase (PME) activity was determined by the method of Hagerman and Austin (1986). Samples of crude enzymes (0.1 mL) was added to a reaction mixture contained the following: 1.0 mL pectin solution (0.01%), 0.2 mL NaCl (0.05 M), 0.1 mL bromothymol blue solution and 0.2 mL distilled water in a cuvette. After that, the cuvette was shaken gently and the absorbance was immediately measured at 620 nm using a spectrophotometer. After 3 min, the absorbance was measured again. The difference in the absorbance was measured as the PME activity. The activity of the PME was calculated using D-galacturonic acid standard curve.

Pectinlyase (PL) and pectate lyase (PNL) activities were assayed by the thiobarbituric acid method (Olutiola and Akintunde, 1979). Samples (0.2 mL) of culture filtrates were added to 1.8 mL of 1.0% pectin dissolved in 0.05M sodium citrate buffer pH 4.8 and the mixtures were incubated for at 40 °C for 30 min. The reactions were stopped by the addition of 1.5 mL of 1 N HCl and 3 mL of 0.04 M thiobarbituric acid into the reaction mixtures. The reactions were kept at 100 °C for 15 min and the absorbance was read at 550 nm afterwards. One unit of lyase activity was defined as the amount of enzyme causing an absorbance change of 0.01 under the described conditions.

2.5 Cellulases enzyme assay

Filter paperase (FPase) was determined by measuring the released of reducing sugar from the filter paper. The crude enzyme (0.2 mL) was mixed with 1 x 6 cm (Whatman No. 1) filter paper strip (3 × 1 cm) and immersed in 1.8 mL of 50 mM sodium citrate buffer (pH 4.8). It was then incubated at 40 °C for 1 hour. The absorbance was measured at 575 nm using spectrophotometer. Activity of FPase was determined by DNS method (Miller, 1959) against standard curve of glucose. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of reducing sugar per minute under standard assay conditions.

2.6 Protein assay

Protein concentration in the culture filtrates was estimated by Bradford method (Bradford, 1976). Protein content was determined by adding 100 µL of sample to 3.0 mL of Bradford reagent. Absorbance was measured at 595 nm using UV visible spectrophotometer. Protein concentration in the unknown samples were calculated from standard curve of Bovine Serum Albumin and expressed as mg protein per mL. As for the blank, 1 mL of sample solution was replaced with 1 mL distilled water.

2.7 Mycelium dry weight

Pre-weighed Whatman No. 1 filter paper was used to filter mycelium. Dry weight of mycelium was measured after drying to a constant weight at 80 °C. The dry weight of mycelium was calculated based on equation below:

$$\text{Dry cell weight} = \frac{(Dried\ \ filter\ \ paper + mycelium\ ) - (dried\ \ filter\ \ paper\ )(g)}{volume\ \ of\ \ the\ cell\ \ (mL)}$$

III. RESULTS AND DISCUSSION

In the present study, pectinolytic and cellulolytic enzymes production by *C. truncatum* CP2 was characterized in synthetic medium containing pectin as carbon source with the *C. gloeosporiodes* served as a control. Fig.1 to Fig.5 shows the growth

and pectinolytic enzyme production by both pathogens. Based on the results of the present study, both of the pathogens were able to grow in the medium containing pectin and produced pectinolytic enzymes. In general, higher pectinolytic enzymes (PG, PME, PL and PNL) and mycelium growth were produced by *C. truncatum* CP2 compared to *C. gloeosporiodes*(Figure1). Maximal growth value of 1.78±0.18 mg/mL was obtained by *C. truncatum* CP2 (8th day of fermentation) which was about 2-fold higher than obtained by *C. gloeosporiodes.*

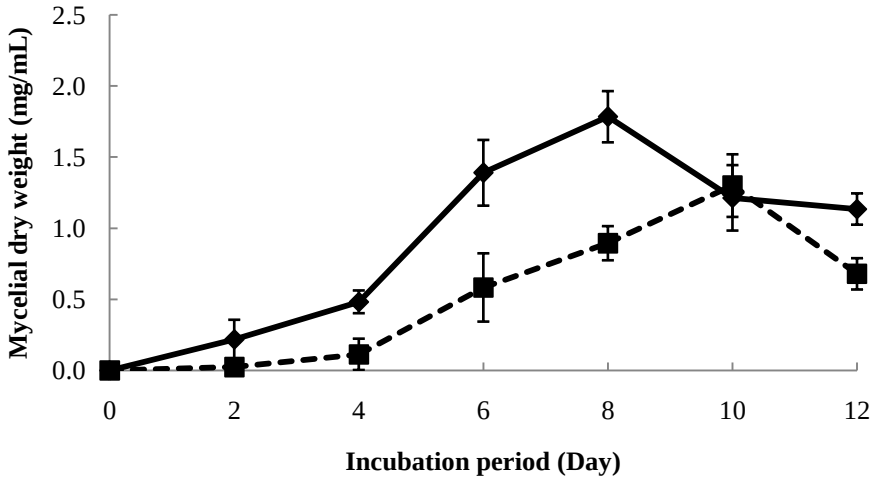

FIGURE 1: GROWTH PATTERNS OF *C. TRUNCATUM* CP2 (♦) AND *C. GLOEOSPORIODES* (■) IN MEDIUM CONTAINING PECTIN FROM CITRUS AS A CARBON SOURCE. VALUES ARE MEANS OF 3 REPLICATES WITH ± SD.

Higher production of PG enzyme was produced by *C. truncatum* CP2 after 6th day of fermentation (0.24±0.10 U/mL) which was earlier compared to other enzymes. The PG activity trend was similar to that *C. gloeosporiodes* cultivated in the medium containing pectin from citrus as carbon source (Figure 2).The activity of PME was almost constant throughout the infection process except a slight increase at day 8, which suggested that the PME plays a little role after formation of pectic substances (Figure 3). Data on PL (Figure4) and PNL (Figure5) of *C. truncatum* CP2 revealed that these enzymes increased throughout the infection process and reached their highest level following the day of maximum growth in which 1.80±0.12 U/mL and 32.7±1.23 U/mL were obtained after 8th day of fermentation, respectively. In comparison, *C. truncatum* CP2 shows higher production of PME, PL and PNL than *C. gloeosporiodes.*

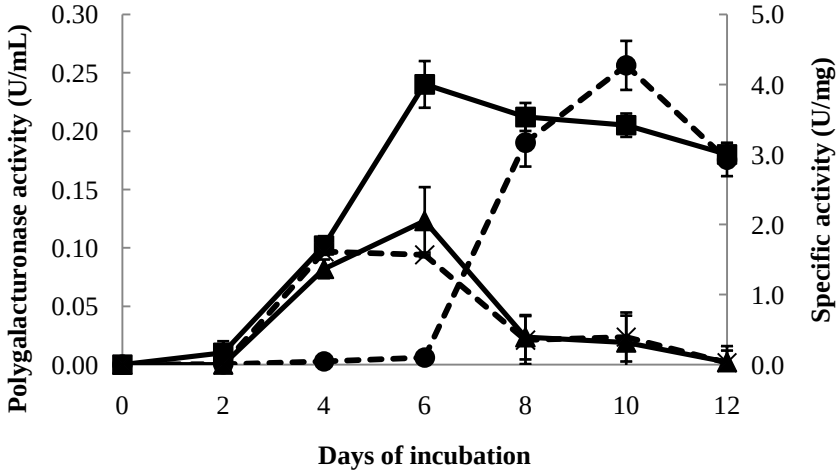

FIGURE 2: PRODUCTION OF PG BY *C. TRUNCATUM* CP2 (■) AND *C. GLOEOSPORIODES* (▲) USING PECTIN FROM CITRUS AS A CARBON SOURCE. VALUES ARE MEANS OF THREE REPLICATES ± SD. SYMBOLS REPRESENT (●) SPECIFIC ACTIVITY FOR *C. TRUNCATUM* CP2 AND (×) SPECIFIC ACTIVITY FOR *C. GLOEOSPORIODES*.

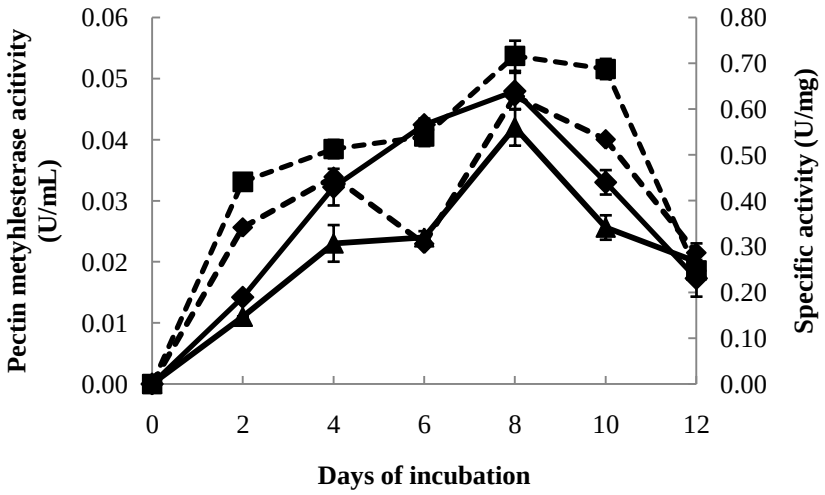

FIGURE 3: PRODUCTION OF PME BY *C. TRUNCATUM* CP2 (♦) AND *C. GLOEOSPORIODES* (▲) USING PECTIN FROM CITRUS AS A CARBON SOURCE. VALUES ARE MEANS OF THREE REPLICATES ± SD. SYMBOLS REPRESENT (●) SPECIFIC ACTIVITY FOR *C. TRUNCATUM* CP2 AND (■) SPECIFIC ACTIVITY FOR *C. GLOEOSPORIODES*.

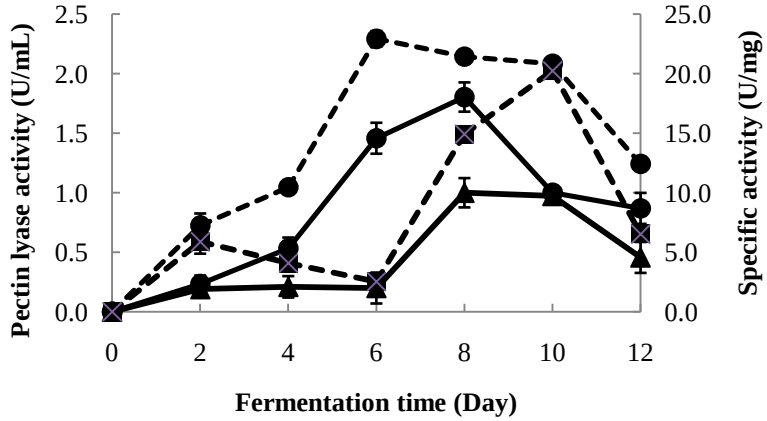

FIGURE 4: PRODUCTION OF PL BY *C. TRUNCATUM* CP2 (●) AND *C. GLOEOSPORIODES* (▲) USING PECTIN FROM CITRUS AS A CARBON SOURCE. VALUES ARE MEANS OF THREE REPLICATES ± SD. SYMBOLS REPRESENT (○) SPECIFIC ACTIVITY FOR *C. TRUNCATUM* CP2 AND (×) SPECIFIC ACTIVITY FOR *C. GLOEOSPORIODES*.

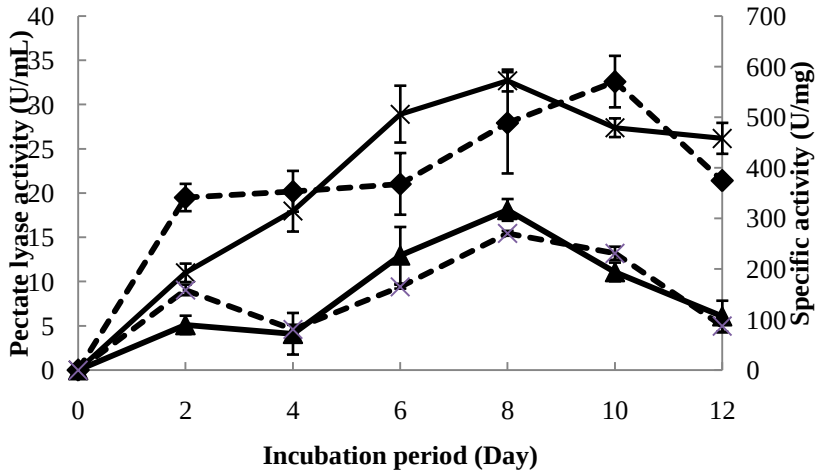

FIGURE 5: PRODUCTION OF PNL BY *C. TRUNCATUM* CP2 (□) AND *C. GLOEOSPORIODES* (▲) USING PECTIN FROM CITRUS AS A CARBON SOURCE. VALUES ARE MEANS OF THREE REPLICATES ± SD. SYMBOLS REPRESENT (♦) SPECIFIC ACTIVITY FOR *C. TRUNCATUM* CP2 AND (×) SPECIFIC ACTIVITY FOR *C. GLOEOSPORIODES*.

The ability of *C. truncatum* CP2 and *C. gloeosporiodes* to produce cellulases enzymes was investigated in this study. Both of these fungal pathogens were able to grow in the media containing CMC as a carbon source. However, the production of cellulase by *C. truncatum* CP2 was much lower than *C. gloeosporiodes* (Fig.6). FPase activity detected from *C. truncatum* CP2 was about 1.85±0.21 U/mL while maximum FPase activity was observed in *C. gloeosporiodes* was 2.19±0.40 U/mL.

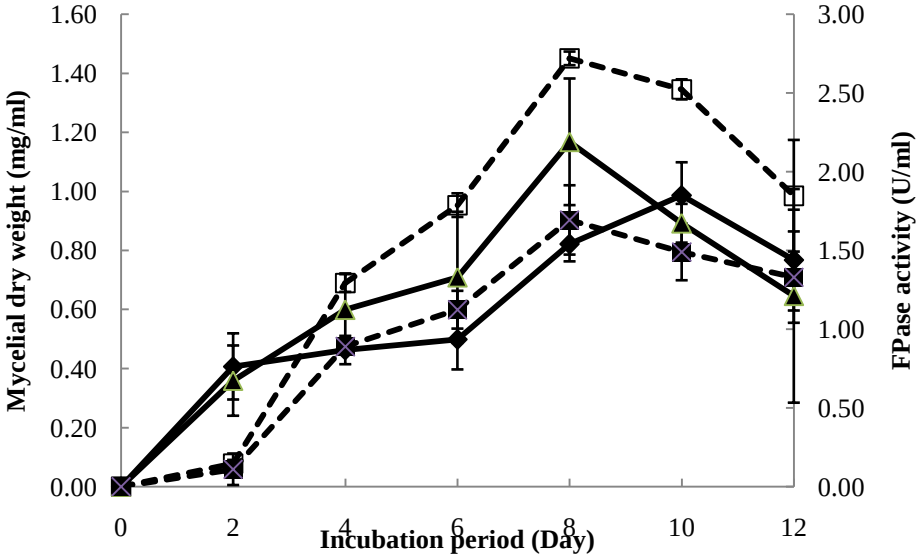

FIGURE 6: PRODUCTION OF FPASE BY *C. TRUNCATUM* CP2 (♦) AND *C. GLOEOSPORIODES* (▲) USING CMC AS A CARBON SOURCE. VALUES ARE MEANS OF THREE REPLICATES ± SD. SYMBOLS REPRESENT (■) SPECIFIC ACTIVITY FOR *C. TRUNCATUM* CP2 AND (○) SPECIFIC ACTIVITY FOR *C. GLOEOSPORIODES*.

The plant cell wall is a major barrier to the establishment of fungal infection on a host. Most plant-pathogenic fungi produce a number of cell wall-degrading enzymes when grown in liquid culture containing pectin. One of these enzymes, PG has been implicated routinely in facilitating the invasion and colonization of host tissue during pathogenesis of fungal pathogens (Choi *et al.*, 2013). The results of the present study demonstrated that both of the *Colletotrichum* species have the ability to produce pectinolytic and cellulolytic enzymes. The early secretion of PG by C. *truncatum* CP2 allows the breakdown of pectic substances. For most necrotrophic fungi, large amounts of pectinolytic enzymes are typically secreted first because it is the only cell wall-degrading enzymes capable of macerating plant tissues and killing plant cells (D'Ovidio *et al.* 2004). PG activity obtained in this work is comparable with other phytopathogenic fungi when using pectin as a carbon source. Earlier, Fernandez*et al.*(1993)reported maximum PG activity of 0.4 U/mLby *Fusarium oxysporum* when grow in pectin whereas about 0.24 U/mL of PG activity was produced by *C. lindemuthianum* was reported by Hugouvieux*et al*. (1997).

The difference observed in the production of pectinolytic enzymes (PG, PME, PL and PNL) activities can be related to the specific role of these enzymes during pathogenesis. The earlier secretion of PG indicates that this enzyme is the most important pectinolytic enzymes that initiates the process of cell wall degradation and facilitates penetration of the pathogen into the host tissues. This result is in close agreement with Oeser*et al*. (2002) who reported that endoPG is the pathogenicity factor in the *Clavisepspurpurea*/rye interaction. In contrast, higher PL and PNL activities appear later which correlated with the necrotrophic phase of tissue colonization. The sequential secretion of these cell wall-degrading enzymes indicates that the pectinolytic enzymes are required to enhance the accessibility of cell wall components for degradation by other enzymes such as cellulases.The contribution of PG, PME and PL to the pathogenicity of some fungal pathogenshas been studied by gene disruption or replacement experiments. For example, the *pelB* mutant of *C. gloeosporiodes* had shown 36-45% reduction in rotting diameter of avocado compared to the wild type isolate which indicate the important role of PL on the pathogenicity of *C. gloeosporiodes* on avocado fruits (Yakoby *et al.*, 2001). Valette-Collet *et al.* (2003) reported that pathogenicity tests of a *Bcpme1* disrupted mutant obtained from *Botyriscinerea* on *Arabidopsis thaliana* and apple fruit showed reduction in rotting activities compared to the wild-type strain, which suggested that PME enzyme play a major role in pathogenesis of *B. cinerea*.

Cellulose is a major polysaccharides present in the plant cell wall and microorganism therefore require specific enzymes for the degradation of cell wall. A number of plant-pathogenic fungi produced cellulolytic enzymes capable of hydrolysing cellulose and its derivatives (Laine *et al*., 2000; Wanjiru *et al*., 2002).The results obtained in the present study indicate that

pathogens *C. truncatum* strain CP2 produced cellulolytic enzymes which able to degrade CMC and the activity increased in age of the culture. The involvement of cellulases in pathogenicity of plant–pathogenic fungi has been demonstrated in previous studies (Babalola, 2010). Eshel *et al*. (2002) reported on secretion of endocellulase by *Alternariaalternata* which directly influenced the disease development in persimmon fruit.

In this study, the pectinolytic and cellulolytic enzymes activity detected in vitro indicate the involvement of these enzymes in development of disease by *C. truncatum* CP2 in chilli fruits. The secretion of pectinolytic and cellulolytic enzymes give ability for this phytopathogenic fungus to invade host tissues which differ in their polysaccharide cell wall composition (Bellincampi *et al*., 2014). Many previous studies have reported on fungal pathogens such as *C. capsici* which secrete both cellulolytic and pectinolytic enzymes to invade the host plant cell walls (Anand *et al.*2008). Similar results were also reported by (Acosta-Rodríguez *et al.*, 2005).

IV. CONCLUSION

In conclusion, cellwall-degrading enzymes likely to be involved in cellwall degradation during surface host penetration and invasion are both pectinases and cellulose. PG was the most important enzyme in initiating the process of cellwall degradation. Once the cellwall was degraded by the action of PG, further degradation of the cellwall was influenced by PME, PL, PNL and cellulases. The variation in enzymatic activity at different intervals may suggest their specific role for pathogenesis at proper timings.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Higher Education Malaysia (Grant No. FRGS/2//2013/SG05/UPM/02/21) for financial support.

REFERENCES

[1] Acosta-Rodríguez, I., Piñón-Escobedo, C., Zavala-Páramo, M. G., López-Romero, E., & Cano-Camacho, H. (2005).Degradation of cellulose by the bean-pathogenic fungus Colletotrichumlindemuthianum.Production of extracellular cellulolytic enzymes by cellulose induction. *Antonie Van Leeuwenhoek*, *87*(4), 301-310.

[2] Anand, T., Bhaskaran, R., Gandhi Karthikeyan, T., Rajesh, M., &Senthilraja, G. (2008).Production of cell wall degrading enzymes and toxins by Colletotrichumcapsici and Alternariaalternata causing fruit rot of chillies. *Journal of plant protection research*, *48*(4), 437-451.

[3] Babalola, O. O. (2011). Pectinolytic and cellulolytic enzymes enhance Fusarium compactum virulence on tubercles infection of Egyptian broomrape. *International journal of microbiology*, *2010*.

[4] Bellincampi, D., Cervone, F., &Lionetti, V. (2014).Plant cell wall dynamics and wall-related susceptibility in plant–pathogen interactions. *Frontiers in plant science*, *5*.

[5] Choi, J., Kim, K. T., Jeon, J., & Lee, Y. H. (2013). Fungal plant cell wall-degrading enzyme database: a platform for comparative and evolutionary genomics in fungi and Oomycetes. *BMC genomics*, *14*(5), S7.

[6] D'Ovidio, R., Mattei, B., Roberti, S., &Bellincampi, D. (2004).Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant–pathogen interactions. *BiochimicaetBiophysicaActa (BBA)-Proteins and Proteomics*, *1696*(2), 237-244.

[7] Eshel, D., Miyara, I., Ailing, T., Dinoor, A., &Prusky, D. (2002).pH regulates endoglucanase expression and virulence of Alternariaalternata in persimmon fruit. *Molecular plant-microbe interactions*, *15*(8), 774-779.

[8] Laine, M. J., Haapalainen, M., Wahlroos, T., Kankare, K., Nissinen, R., Kassuwi, S., & Metzler, M. C. (2000). The cellulase encoded by the native plasmid of Clavibactermichiganensis ssp. sepedonicus plays a role in virulence and contains an expansin-like domain. *Physiological and Molecular Plant Pathology*, *57*(5), 221-233.

[9] Leone, G. (1992). Significance of polygalacturonase production by Botrytis cinereain pathogenesis. *Recent advances inBotrytisresearch*, 63.

[10] Niture, S. (2008).Comparative biochemical and structural characterizations of fungal polygalacturonases. *Biologia*, *63*(1), 1-19.

[11] Rogers, L. M., Kim, Y. K., Guo, W., González-Candelas, L., Li, D., &Kolattukudy, P. E. (2000). Requirement for either a host-or pectin-induced pectate lyase for infection of Pisumsativum by Nectriahematococca. *Proceedings of the National Academy of Sciences*, *97*(17), 9813-9818.

[12] Saxena, A., Raghuwanshi, R., Gupta, V. K., & Singh, H. B. (2016). Chilli anthracnose: The epidemiology and management. *Frontiers in microbiology*, *7*.

[13] Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., &Boccara, M. (2003). Disruption of Botrytis cinerea pectin methylesterase gene Bcpme1 reduces virulence on several host plants. *Molecular Plant-Microbe Interactions*, *16*(4), 360-367.

[14] Wanjiru, W. M., Zhensheng, K., &Buchenauer, H. (2002).Importance of cell wall degrading enzymes produced by Fusarium graminearum during infection of wheat heads. *European Journal of Plant Pathology*, *108*(8), 803-810.

[15] Yakoby, N., Beno-Moualem, D., Keen, N. T., Dinoor, A., Pines, O., &Prusky, D. (2001).Colletotrichumgloeosporioidespelb is an important virulence factor in avocado fruit-fungus interaction. *Molecular Plant-Microbe Interactions*, *14*(8), 988-995.

Wood density variation of different provenance for exotic loblolly pine in China

Xu –Youming[1], Lin-Han[2], Zhen-Hongpo[3], Liu-Jihong[4], Zhou-Caixia[5]

College of Forestry and Horticulture, Huazhong Agricultural University, Wuhan 430070, P R China
Corresponding Authors: Dr Prof. Youming Xu
College of Forestry and Horticulture, Huazhong Agricultural University, Wuhan 430070, Hubei Province, P R China
Fax: 86-27-87282010
Tel: 86-27-87282010 (O), 86-18971560337
E-mail: xuyouming@mail.hzau.edu.cn

Abstract— *Variation patterns and range of juvenile wood basic density (BD) of 30 loblolly pine provenances in Zhejiang Province of China were reported in this paper. There were four radial variation patterns of BD within 310 10-year-old trees of 30 provenances, but the main pattern was a steadily increasing trend from pith to bark. The early-late correlation coefficient of BD for loblolly pine strengthens with trees age and the value of BD in 5 years old trees can be used for predict that in 10 years old trees. The BD mean change of 30 provenances is similar to normal distribution and the BD varies from 0.343 to 0.412 g/cm3. The BD mean of the contrast provenance is 0.369 g/cm3 and in medial level. There is an obvious difference in BD among 30 provenances. Further analyses show that the difference among trees within a provenance is greater than that among provenances and the environment has significant effects on its BD. The broad heritability of BD is 0.72. The individual tree selection within a provenance was much better than that among different provenance.*

The BD mean of 30 provenances is related positively to the provenance latitude, Longitude of provenance is related negatively to wood density in East Coast and positive to wood density in South Coast and Gulf. There is a tendency for BD of loblolly pine provenance to be lower from west to east and from north to south. This variation in plantation in a common environment turned out contrary to that of natural at different environment. The BD of loblolly pine provenances introduced from high latitude is greater than that from low latitude, but tree growth properties from low latitude were much faster than those from high latitude.

The BD mean value of 310 trees at the same growth stage (the same age) was negatively related to the diameter at breast height and volume of these trees. Wood density was significant correlated negatively with tracheid width and tracheid diameter at different growth stage of loblolly pine. DBH was significant positively correlated with tracheid width, tracheid diameter and negatively distinct related to ratio of tracheid double wall thickness to tracheid diameter. That DBH was related positively to ratio of tracheid diameter to tracheid width and negatively to tracheid wall thickness showed that the tracheid wall thickness of fast trees or fast provenances did not increase in proportion as tracheid width and tracheid diameter increased. This would lead to the decrease in wood basic density. Better provenance selection for pulpwood and building lumber should be determined from both tree growth and wood properties.

Keywords— loblolly pine, wood density, variation, wood anatomy, provenance trial and correlation analyses.

I. INTRODUCTION

Pinus taeda L originated from the southeastern of the U. S. A. is one of the most important exotic species for wooden industrial materials in subtropical area of the world. It was first introduced to plant in China in 1933 (Peng, 1992). Now it is extensively planted on hilly lands and low mountains in the Southern China for pulpwood and building lumber. It was estimated that the areas of its plantation is at least more than one million hectares in 1995. Most of these stands for loblolly pine are 20~25 years old and do not look good in both its stem shape and wood quality. It is necessary for them to be replaced with better provenances of loblolly pine within 10~15 years.

Although loblolly pine has been culturing in China for more than seventy years, its provenance trial was not formally done until 1981 (Peng, 1992). According to the statistics in 1992 its seed orchard in China was about 809 hectares and its seed stand about 2720 hectares. Every year about 25000kg of seeds was imported from the American (Peng, 1992). The seed output is far from the demand of our forestry production and the genetic quality of seed is low. Because its natural growing range in the American is large and there are significant differences in its tree growth, resistance and wood quality among the seed sources (Zobel 1958 and 1989), it is significant to for us to select better provenance for tree growth and wood improvement (Liu 2010; Kimura 2014; Sharma 2015; Takeuchi 2016).

In the United States wood properties of loblolly pine usually follow a rather predictable pattern (Koch 1972 and Zobel 1989). The inland and northern sources of loblolly pine have lower specific gravity and frequently shorter tracheid length than southern and coastal sources (Jackson and Strickland 1962, Mitchell 1964, Talbert and Jett 1981, Zobel 1958 and 1989). But the studies carried out by Byram (1980), Tauer (1990), Jayawickrama, Mckeand and Jett (1997) showed that wood properties of loblolly pine did not follow the rather predictable patterns. These showed the environment had a significant effect on variation of wood basic density for loblolly pine (Zobel 1989). What is about variation of wood basic density for loblolly pine provenance in China? Is there a rather predictable pattern in its wood basic density in China? We would like to know these answers. The provenance trial of loblolly pine organised by Chinese Academic of Forestry was carried out two times in 1981 and 1983 in China, respectively. The seeds for experiment in 1981 were collected from the natural stands and for experiment in 1983 were obtained from the improved seeds orchard of loblolly pine in United States (Peng 1992).

Wood density is an important index for both wood properties and wood improvement in tree breeding (Panshin 1980; Zobel 1984; Liu 2010; Kimura 2014; Sharma 2015; Takeuchi 2016). Its variation will affect wood quality, pulp yield per hectare, quality of paper and pulp and wood products. The objectives of this paper will study the variation patterns of wood basic density for loblolly pine on the bases of its provenance trial in 1983 in China. The results obtained will supply the theoretical bases in wood improvement of loblolly pine, its plantation culturing and reasonable and efficient utilizing of its wood resources.

II. MATERIALS AND METHODS

2.1 Test materials

The trial stand of 31 loblolly pine provenances was established in spring of 1993 in the Forest Farm of Fuyang County in Zhejing Province (in eastern of China). The Forest Farm is located at 119°58' E and 30°15' N. Its annual rainfall is 1478mm and means temperature is 15.6°c. Its frostfree season is 221 days per year. The test stand is located at the hill of about 150m ~170m height above sea level in the Forest Farm. Its site is red soil and within 60~80cm. The pH was about 5.1.

The field plan of provenance was designed in form of randomized block, double lines in a block are twenty trees and replication number is five. Plant and row spacing in the test stand is 1.5×2.5 m^2. The seeds of 30 provenances provided by Forest Service of the American were separately collected from improved stands of twenty states in the southeastern of the American. The seeds collected from trees of loblolly pine planted in Wuhan in 1945 were tested together as a contrasting material. The geographical and meteorological factors of various provenances in their original regions were presented in Table 1.

TABLE 1
THE ORIGIN AND THE LOCAL FACTORS OF GEOGRAPHY AND METEOROLOGY OF 31 PROVENANCE

Prov No	Geographic Location	Latitude	Longitude	Prov No	Geographic Location	Latitude	Longitude
1 CL-3	Maryland Worcester	38°15'	75°30'	17 RL-3	Alabama Claoctaw	32°	88°
2 CL-2	Virginia King & Queen	37°30'	77°	18 RL-2	Alabama Buter	31°30'	86°45'
3 RL-1	Virginia New Kent	37°30'	77°	19 RL-1	Alabama Dallas	30°30'	87°
4 CL-8	Kentucky Ballard	37°	89°	20 RL-8	Alabama Monreo	31°30'	87°
5 RL-2	North Carolina Gates	36°30'	77°	21 RL-2	Alabama Pickens	33°	88°
6 RL-3	North Carolina Anson	35°	80°	22 RL-3	Mississippi Jones	31°30'	89°
7 RL-5	North Carolina Wayne	35°	78°	23 RL-5	Mississippi Franklin	31°30'	91°
8 RL-6	South Carolina Georgetown	34°	79°	24 RL-6	Louisiana Livigston	30°30'	91°
9 RL-8	South Carolina Greenwood	34°15'	82°15'	25 RL-8	Louisiana Grant Parish	31°30'	92°
10 RL-9	South Carolina Sumter	34°	80°30'	26 RL-8	Louisiana Desoto	32°	94°
11 RL-11	Georgia Bibb	32°30'	83°45'	27 CL-8	Texas Jasper	36°	90° 30'
12 RL-12	Georgia Stewart	32°	84°45'	28 CL-8	Arkansas Pike	34°	93°
13 RL-14	Georgia Evans	32°0	82°	29 CL-8	Texas Marion	33°	95°
14 RL-20	Geogia Margan	33°30'	83°30'	30 CL-8	Texas Jasper	31°	94° 30'
15 RL-16	Florida Nassau	30°30'	81° 30'	31 CK	Wuhan of China	30° 31'	114° 29'E
16 RL-17	Florida Marion	29°	82°	Trial location		30° 31'	119°58'E

2.2 Methods

The trial stand's age of loblolly pine was 10 years old in 2003. At this time it was necessary for the trial stand to be thinned. The height and diameter at breast height of each tree in the stand were first measured before thinning. Then ten trees of each provenance from the trial stand (two trees per replication in each block) were selected to use as test material. After trees were cut down the diameter of 1/2 height of each tree was measured. Based on the increments the volume of each tree was

calculated. A round-timber about 2.7m started at 1.2 m height from base to top was sawn from each tree. Totally 310 trees were collected for 31 provenances.

In the laboratory five clear sampled discs of about 2~4cm in thickness were sawn near the breast height from a round-timber. These discs were used for measuring features of wood anatomy, basic density, shrinkage of wood and chemical composition of wood. 3~5 test specimens of each wood property were cut from pith to bark at each disk on the condition of the same ring age range. Measurements of ring width and latewood width were taken directly on the smooth discs processed with the help of a stereomicroscope fitted with a micrometer. Based on the measurement the latewood percentage could be calculated. Basic density of 1~3 rings, 4~5 rings and 6~9 rings were determined by the maximum moisture method of Smith in 1954, respectively.

After determination of basic density the wood of 1~3 rings, 4~5 rings and 6~9 rings was used for determining their tracheid anatomic features. First specimens of earlywood and latewood were macerated with Juffery's fluid respectively. Then tracheids were directly measured by projecting them on a screen (40cm diameter) at 50X or 100X magnification with a 35mm slide projector. Thirty readings were made per sample, selecting randomly unbroken tracheid. The values of tracheid width, tracheid diameter and tracheid wall thickness were acquired directly on 20um thick cross microtome section with microscope at 400X magnification, selecting thirty cells separately from earlywood to latewood to be determined.

The specific weighted mean values for the 1~5 rings and the 1~9 rings in tracheid anatomic features were calculated on the bases of the mean values measured of 1~3 rings, 4~5 rings and 6~9 rings according to the percentages of their rings width in total width of the rings range.

With the SAS, the difference of wood basic density among provenances of loblolly pine was analyzed and the relationships between basic density and anatomic characteristics of provenances to their geographic and meteorology factors were assessed.

III. RESULTS AND ANALYSES

3.1 Radial variant pattern of trees within a provenance of loblolly pine

TABLE 2
VARIANT PATTERNS OF WOOD BASIC DENSITY WITHIN TREES FOR EXOTIC LOBLOLLY PINE PROVENANCE

Prov. No	Pattern I No %	Pattern II No %	Pattern III No %	Pattern IV No %	Prov. No	Pattern I No %	Pattern II No %	Pattern III No %	Pattern IV No %
1	6 60	2 20	2 20	0 0	17	6 60	0 0	4 40	0 0
2	5 50	0 0	4 40	1 10	18	7 70	1 10	2 20	0 0
3	7 70	3 30	0 0	0 0	19	5 50	4 40	1 10	0 0
4	7 70	1 10	2 20	0 0	20	5 50	2 20	3 30	0 0
5	9 90	1 10	0 0	0 0	21	8 80	0 0	2 20	0 0
6	7 70	0 0	3 30	0 0	22	7 70	2 20	1 10	0 0
7	6 60	0 0	4 40	0 0	23	7 70	0 0	3 30	0 0
8	7 70	2 20	1 10	0 0	24	8 80	1 10	1 10	0 0
9	6 60	0 0	4 40	1 10	25	10 100	0 0	0 0	0 0
10	6 60	1 10	3 30	0 0	26	7 70	1 10	2 20	0 0
11	4 40	3 30	3 30	0 0	27	8 80	1 10	1 10	0 0
12	10 100	0 0	0 0	0 0	28	9 90	0 0	0 0	1 10
13	7 70	2 20	1 10	0 0	29	8 80	1 10	1 10	0 0
14	8 80	0 0	2 20	0 0	30	6 60	1 10	2 20	1 10
15	9 90	0 0	0 0	1 10	31	7 70	0 0	2 20	1 10
16	8 80	1 10	1 10	0 0	Total	220 70.9	29 9.4	55 17.7	6 1.9

The results showed that there were four kinds of radial variant patterns within trees of 31 peovenancs for exotic loblolly pine. In Table 2, the pattern I was wood basic density first increasing from pith to bark within trees. The possibility range of the pattern I that appeared was from 40% to 100% with a provenance and its mean possibility was 71 %. The pattern II was basic density first increasing from pith to bark within trees, then decreasing; The possibility range of the pattern II which appeared was from 10% to 40% with a provenance and its mean possibility was 9.4 %. The pattern III was first decreasing slightly in radial direction, then increasing and the possibility range of the pattern III which appeared was from 10% to 40% with a provenance and its mean possibility 17.7 %. The pattern IV was decreasing in radial direction and its mean possibility appeared was 1.9%. It was seen that the main patterns were I and III. Therefore there were 71% to 88.6% possibility that variation of basic density in radial direction for juvenile loblolly pine increased from pith to bark, which was in accordance with the radial variation pattern of specific gravity within trees of loblolly pine conducted by Loo and McNee in 1985. The

mean value of wood basic density for 1~3 rings in redial direction on the 300 discs of 30 loblolly pine (except the Prov. 31) was 0.339g/cm^3, the 4~5 rings was 0.377g/cm^3 and the 6~7 rings was 0.419 g/cm^3. Totally basic density showed increasing pattern and unstable from pith to bark within trees. Because there are four radial patterns for wood density in stands of juvenile loblolly pine and variation of its wood density is unstable, the number of specimens and the ring age range are important in order to get the right result in wood improvement.

3.2 Early – late relationship of wood basic density for loblolly pine at different growth stage

The short rotation of loblolly pine plantation for pulpwood is about 10~15 years and its rotation for building lumber is about 25~30 years in China. It is significant for us to know the early–late relationship of its wood basic density at different growth stage in wood improvement. In table 3 relationship coefficients of wood basic density between the 3-year-old trees and 5-year-old trees for 30 provenances were from 0.1167 to 0.9514. Relationship coefficients of 26 provenances were found to be statistically significant at the 95 percent level of confidence ($r_{0.05}$ = 0.6030, n = 10) and their proportions were 76.7% within 30 provenances. The relationship coefficients of wood basic density between the 3-year-old trees and 9-year-old trees were from 0.2994 to 0.9356 for 30 provenances. 22 provenances were statistically significant at the 95 percent level of confidence and their proportions were 73.7%. The relationship coefficients of wood basic density between 5-year-old trees and 9-year-old trees for 30 provenances were from 0.6515 to 0.9579. All provenances were found to be statistically significant at the 95 percent level of confidence and 24 provenances were statistically significant at the 99 percent level of confidence. These reflected that the relationship property of wood basic density for loblolly pine was strengthened as tree age. Hence the value of wood basic density for 5-year-old loblolly pine could be used as a predicting index of 10-year-old loblolly pine for pulpwood.

TABLE 3

EARLY-LATE RELATIONSHIP COEFFICIENTS OF WOOD BASIC DENSITY FOR LOBLOLLY PINE PROVENANCE AT DIFFERENT GROWTH STAGE

Prov. No.	Early age	Late estimated age of trees		Prov. No.	Early age	Late estimated age of trees	
		5 –year-old	9-year-old			5 –year-old	9-year-old
1	3-year-old 5-year-old	0.7377*	0.8383*** 0.9738***	16	3-year-old 5-year-old	0.8913***	0.8041** 0.9255***
2	3-year-old 5-year-old	0.4105	0.5743 0.9035***	17	3-year-old 5-year-old	0.7665**	0.8499*** 0.9698***
3	3-year-old 5-year-old	0.6621*	0.6570* 0.9665***	18	3-year-old 5-year-old	0.6395*	0.8618*** 0.8978***
4	3-year-old 5-year-old	0.8741***	0.8530*** 0.9727***	19	3-year-old 5-year-old	0.8481***	0.8777*** 0.9759***
5	3-year-old 5-year-old	0.8280***	0.4501 0.9645***	20	3-year-old 5-year-old	0.5093	0.7981*** 0.8913***
6	3-year-old 5-year-old	0.7260***	0.2994 0.8385***	21	3-year-old 5-year-old	0.5790	0.6946* 0.9448***
7	3-year-old 5-year-old	0.3794	0.4276*** 0.9051***	22	3-year-old 5-year-old	0.6032	0.6747* 0.9257***
8	3-year-old 5-year-old	0.8029**	0.9074*** 0.8997***	23	3-year-old 5-year-old	0.7085**	0.7794** 0.9571***
9	3-year-old 5-year-old	0.8742***	0.8994*** 0.9795***	24	3-year-old 5-year-old	0.6537*	0.8446*** 0.9251***
10	3-year-old 5-year-old	0.9514***	0.9356*** 0.9727***	25	3-year-old 5-year-old	0.6442*	0.3423 0.8757***
11	3-year-old 5-year-old	0.1167	0.5540 0.6515*	26	3-year-old 5-year-old	0.8417***	0.8763*** 0.9721***
12	3-year-old 5-year-old	0.8701***	0.7760** 0.9377***	27	3-year-old 5-year-old	0.3384	0.5078 0.8968***
13	3-year-old 5-year-old	0.9088***	0.8906*** 0.9550***	28	3-year-old 5-year-old	0.6721*	0.9135*** 0.8801***
14	3-year-old 5-year-old	0.4002	0.4762 0.9560***	29	3-year-old 5-year-old	0.8419***	0.8464*** 0.8583***
15	3-year-old 5-year-old	0.7746***	0.7053*** 0.9549***	30	3-year-old 5-year-old	0.7360**	0.6488* 0.9198***

3.3 The difference analyses of wood basic density among provenances

Variance analyses in Table 4 showed that there was an obvious difference in wood basic density among 31 provenances for loblolly pine. According to the simple equation ($h_f^2 = (1-1/F)^{1/2}$ of broad sense heritability, $h_f^2 = 0.7215$. These showed that provenance selection in this trial was effective. Of 31 provenances in Table 5, basic density of No 26 was the highest (0.416 g/cm^3) and that of No 12 the smallest (0.343 g/cm^3). Their difference was 0.073g/cm^3 and the percentage 29%. Basic density of the contrasting provenance (No.31) was 0.369 g/cm^3 and in middle level. Duncan's multiple range's test in Table 5 indicated the obvious statistically differences in Table 4 existed only among parts of 31 provenance. For most of 31 provenances, their differences were not significant. This could be verified from the value of variation coefficient (C. V.) for 30 provenances in Table 6. For example, the C. V. of No.10 was the maximum and its value 13.5%; The difference in basic density between trees within No.10 provenance was 0.145 g/cm^3. The difference in basic density between trees within No.26 with the highest wood density was 0.105 g/cm^3 and the difference in basic density between trees within No 12 with smallest wood density was 0.055 g/cm^3. The variant range of wood basic density for No.31 (the contrasting material) was from 0.327 g/cm^3 to 0.429 g/cm^3 and the difference was 0.102 g/cm^3. Population mean value of wood basic density for 31 provenance was 0.372 g/cm^3 and its C. V. was only 5.0% among 31 provenances, which was almost less than C. V. between trees of each provenance except No.27 and No.30. These showed that the difference among trees within a given provenance was greater than that among provenance, i.e., there was much larger difference between trees within a provenance. Hence individual tree selection within a better provenance was much better than that among different provenance.

TABLE 4
VARIANCE ANALYSES OF WOOD BASIC DENSITY AMONG 31 PROVENANCE FOR 10-YEAR-OLD LOBLOLLY PINE

Variance source	Df	S. S.	M. S.	F-value	Viarance Proportion (%)	h_f^2
Provenance	30	0.49310	0.0016433	3.60**	43.57	0.7215
Block	4	0.00839	0.0023232		7.89	
Error	120	0.05492	0.0004577		48.54	

Among the total variance in table 4 proportion of provenance variance was 43.57% and that of environment 56.43%. This revealed that environment had a great significance effect on wood density. This showed that planting site and better silviculture practices were important for increasing growth increment and improving wood quality of loblolly pine. In culturing plantation of loblolly pine for pulpwood and building lumber better provenance should be combined together with selecting good site.

3.4 Relationship analyses of wood basic density to latewood percent and trees growth increments

In Fig.1 latewood percent and wood basic density in radial direction within trees increased as trees age. Wood basic density was positively significant related to latewood percent and their correlation coefficient was 0.4905 (n =31, $r_{0.01}$ = 0.4487). These were in accordance with the research results (Pashin 1980 and Zobel 1989). Among provenances correlation coefficient of wood basic density with tree increments were 0.1285 in tree height, -0.4045 in DBH and -0.3535 in tree volume with simple linear model, respectively. It was found that the negative relationships of wood basic density to DBH and tree volume were significant (n=31, $r_{0.05}$ = 0.3493) at the 95 percent level of confidence. For example, the growth increments of provenances of No.10, No.13, No.15, No.22, No.25, No.31 in Table 5 were much better than the rest of 31 provenances, but their wood density were less than the population mean. These indicated that better provenance should be selected from tree growth increment and wood properties because a little change (0.04g/cm^3) of wood density would cause a great change in weight of wood dried per hectare (Zobel 1989). For pulpwood of short rotation the weighted value of tree growth increments could be increased in better provenance selection. To wood structural material wood quality should be much considered in its better provenances selection.

The mean values of wood density, tree height, DBH and tree volume for 31 provenance in Table 5 were 0.372 g/cm^3, 6.47m, 9.68cm and 0.0263m^3 and their weighted values were 0.45, 0.10, 0.20, 0.35 in order. The formula

$$Si = (45*(BDi-0.372)/0.372)+(15*(Hi-6.47)/6.47)+(20*(Di-9.68)/9.68) + (30*(Vi-0.0263)/0.0263)$$

was utilized to calculate the Score of each provenance. If Si of a provenance > 0, it ranked above the middle level. If Si < 0, it ranked below the middle level. The bigger the Si, the better the provenance will be. Finally the provenances of No.15, No.10, No.13, No.31, No.26, No.20 and No.25 as better provenances for pulpwood were selected comprehensively out from tree increment and wood density.

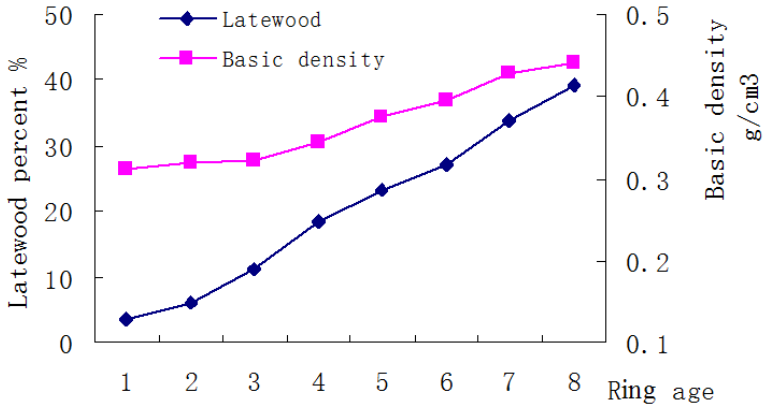

FIG 1: RELATIONSHIP OF WOOD BASIC DENSITY TO LATEWOOD PERCENTAGE

TABLE 5

DUNCAN'S MULTIPLE RANGE TEST AND THE SCORES OF EACH PROVENANCE FORM TREE GROWTH AND WOOD QUALITY

Prov No.	Basic density (g/cm3)	α= 0.05 level	α=0.01 level	Tree height (m)	DBH (cm)	Volume (m^3)	Score
26	0.416	A[1]	A	7.08	9.15	0.0263	5.7*
30	0.412	Ab	Ab	6.63	9.48	0.0236	1.6
27	0.403	Abc	Abc	6.56	8.85	0.0248	0.6
23	0.397	Abcd	Abcd	6.57	9.35	0.0264	2.7
18	0.396	Abcde	Abcd	6.74	9.45	0.0256	- 1.1
19	0.391	Abcdef	Abcd	6.34	8.90	0.0267	0.8
5	0.384	Abcdef	Abcd	6.07	9.30	0.0246	- 2.2
1	0.383	Abcdef	Abcd	6.15	9.07	0.0218	- 5.8
28	0.382	Abcdef	Abcd	6.60	9.25	0.0243	- 1.7
4	0.377	Abcdef	Abcd	6.63	9.58	0.0266	1.2
20	0.377	Abcdef	Abcd	6.57	10.23	0.0286	4.7*
21	0.372	Abcdef	Abcd	6.43	9.23	0.0263	- 1.0
17	0.371	Abcdef	Abcd	6.46	10.10	0.0265	0.5
29	0.370	Abcdef	Abcd	6.67	10.47	0.0295	- 5.5
2	0.370	Abcdef	Abcd	5.55	8.12	0.0169	-16.3
31	0.369	Abcdef	Abcd	6.58	10.95	0.0330	10.1**
24	0.368	Abcdef	Abcd	6.57	9.07	0.0232	- 5.0
10	0.368	Abcdef	Abcd	6.72	11.12	0.0368	15.0**
11	0.365	Abcdef	Abcd	6.30	9.85	0.0232	- 4.4
7	0.365	Abcdef	Abcd	5.79	10.08	0.0245	- 3.6
25	0.364	Abcdef	Abcd	7.00	10.27	0.0288	4.3
3	0.363	Abcdef	Abcd	6.51	8.92	0.0259	- 2.9
6	0.363	Abcdef	Abcd	6.17	9.60	0.0274	- 0.7
8	0.363	Abcdef	Abcd	6.27	9.53	0.0229	- 5.8
22	0.363	Abcdef	Abcd	6.30	9.60	0.0250	- 3.1
9	0.356	Bcdef	Abcd	6.10	9.22	0.0233	- 7.1
13	0.355	Cdef	Abcd	6.82	11.07	0.0347	11.2**
16	0.348	Cdef	Abcd	6.35	10.10	0.0265	- 2.1
14	0.347	Ef	Bcd	6.07	9.52	0.0235	- 7.4
15	0.346	Ef	Cd	7.23	11.90	0.0370	15.2**
12	0.343	f	D	6.61	9.33	0.0237	- 6.9

[1] *Values sharing the same letter are not significantly different at the 0.05 and 0.01 levels.*

3.5 Relationship analyses of wood basic density to the factors of provenance's geography and meteorology in their indigenous regions

Considering the physical features of the USA topography and the distribution of provenances (seen in Fig 2), the natural range areas of loblolly pine were divided into three parts so that the relationship of wood density to the geographic and

meteorological factors of provenances in each part was further analyzed. The provenance number of East Coast, South Coast and Mexico Gulf and Hinterland districts was 15, 14 and 6 separately.

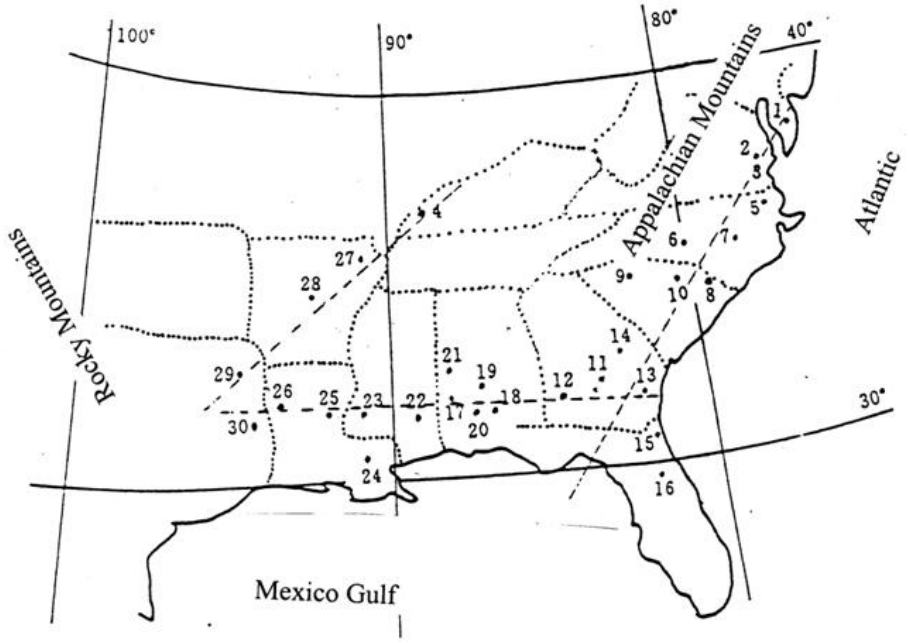

FIG 2: THE LOCAL DISTRIBUTION OF 30 PROVENANCES FOR EXOTIC LOBLOLLY PINE AND THE NUMBER OF THEIR CODE.

Wood density in Table 6 was correlated positively with latitude, annual rainfall and forstfree season, and negatively with longitude and annual mean temperature. This simple linear regression showed that the five local factors of latitude, longitude, annual rainfall and forstfree season for 15 provenances in East Coast districts near Atlantic had significant effects on wood basic density of loblolly pine ($r_{0.01}$=0.6055, n = 15). It was seen that annual mean temperature and forstfree season were main factors of affecting wood density from the magnitude of deviation regression coefficient. Actually this reflected the effect of latitude on wood basic density. This showed that the wood of provenances from higher latitude planted in lower latitude districts like our trial location had higher wood density and the wood from lower latitude had lower wood density. These were in accordance with the research results reported by Jackson (1962), Byram (1988) and Tauer (1990).

TABLE 6

CORRELATION COEFFICIENT OF WOOD DENSITY WITH THE FACTORS OF PROVENANCE'S GEOGRAPHY AND METEOROLOGY IN THEIR LOCAL REGIONS

District	Item	Latitude	Longitude	Annual mean temperature	Annual rainfall	Forstfree season	Notes
East coast	Simple regression	0.7838**	- 0.7893**	- 0.6901**	0.6975**	0.7089**	$r_{0.05}$=0.6055
	Deviation regression	0.2460	- 0.3439	0.3855	- 0.1107	- 0.3857	n=15
South coast and Mexico gulf	Simple regression	0.3554	0.5874*	- 0.4067	- 0.1818	- 0.4901*	$r_{0.05}$=0.4638
	Deviation regression	- 0.1127	0.2765	0.0184	- 0.1917	- 0.2193	n=14
Hinterland	Simple regression	- 0.4710	0.1879	0.5335	0.5230	0.3456	$r_{0.05}$=0.7076
	Deviation regression	0.7813	0.9989	0.3387	- 0.8957	0.9950	n=6

In the districts near South Coast and Mexico Gulf wood density was positive correlated with latitude of provenances and negative with annual mean temperature, which was consistent with the results in East Coast in Table 6. But wood basic density was positively related to longitude of provenances and negatively to annual rainfall and forstfree season, which were obvious different from the results in East Coast in Table 6. These indicated that wood of provenance of higher longitude districts far from East Coast planted near coast line area like our trial location would possess higher wood density, which was consistent with the research results of Byram (1988) and Tauer (1990). In the districts of Hinterland the results reflected the negative effect of povenance's latitude on wood density, i.e., the provenance near Mexico Gulf planted in our trial location

would have higher wood density. Being a few numbers of provenances in Hinterland districts the result could be considered as a reference in its tree breeding.

In a word the local latitude of provenances was pronounced positively correlated with wood density in our trial location. This geographical variation pattern was distinct different from the north to south increase in wood density reported in the literature for stands of natural (Zobel and McElwee 1958, Mitchell 1964) and plantation (Teltbert and Jett 1981) and the northwest to southeast increase (Mitchell 1964), but complete agreement with the research results of plantation stands (Jackson 1962, Byram 1988, Tauer 1990, Jayawickrama 1997). A hypothesis given by Byram for the evident trend in his study was that genetic variation in wood density was controlled not only by the date of growth initiation, but also by the time of transition from springwood to summerwood production. Wood density could be determined by the length and amount of rainfall in the growing season and provenances from Southern districts would begin earlier in the spring. The radial growth cessation occurred at approximately the same time for all provenance and the southern sources made the transition to summerwood production at the same time or later than the northern sources. The southern sources would have a greater amount of springwood in the whole growth ring and a lower wood density. Jayawickrama's experiment showed that fast-growing southern and coastal sources of loblolly pine with lower wood density than northern and inland sources could be explained by a later transition to latewood, associated with a longer period of height growth, of the fast-growth sources.

Our provenances trial was conducted at lower latitude of Zhejiang Province in China. The reasons about wood density variation could exist as their explanation and experiment. But the inside property of the provenance could be other important factors. Usually trees of southern sources and east coastal sources grow faster than northern sources and hinterland. At the same length of growth season and summer the amount of springwood produced in the whole ring for southern and east coastal provenances should be higher than northern and hinterland provenances in spring. If the radial growth cessation occurred at approximately the same time for all provenance or the southern sources made the transition to summerwood production at the same time or later than the northern sources, the growth season of northern and hinterland provenances planted in the lower latitude districts near coastal line (like our trial location) should be longer than their indigenous districts and their latewood percentage should be increased, which would result in increase of wood density.

TABLE 7
CORRELATION COEFFICIENTS OF TEACHEID ANATOMICAL FEATURES WITH DBH OF 310 TREES LOBLOLLY PINE

Tracheid length	Tracheid width	Tracheid diameter	Tracheid wall thickness	Tracheid length / width	Double wall thickness/ diameter	Tracheid diameter / width
- 0.0681	0.3596**	0.3635**	- 0.0725	- 0.2211	- 0.3104**	0.1276

TABLE 8
CORRELATION COEFFICIENTS OF WOOD BASIC DENSITY WITH TRACHEID ANATOMIC FEATURES FOR 310 TREES OF LOBLOLLY PINE PROVENANCE

Tracheid features	1 ~ 3 rings	4 ~ 5 rings	6 ~ 7 rings	1 ~ 5 rings	1 ~ 9 rings
Tracheid length	- 0.1423	- 0.0480	- 0.0220	- 0.0870	- 0.0519
Tracheid width	- 0.2672**	- 0.2758**	- 0.2799**	- 0.2789**	- 0.3833**
Tracheid diameter	- 0.3872**	- 0.3703**	- 0.3487**	- 0.3901**	- 0.4580**
Tracheid length-width ratio	0.0312	- 0.1433**	0.2465**	0.1191	0.1082

Correlation coefficients of DBH of 310 trees for 10-year-old loblolly pine with tracheid anatomical features were given in Table 7. At 99% statistical confidence level testing, DBH was significant positively correlated with tracheid width, tracheid diameter and distinct negatively related with ratio of tracheid double wall thickness to tracheid diameter (n=310, $r_{0.01}$=0.1452). These results reflected that fast growing of provenances or fast growing trees to a great extent attributed to the increase of tracheid width and tracheid diameter. That DBH was positively related to ratio of tracheid diameter to tracheid width and negatively to tracheid wall thickness showed that the tracheid wall thickness of fast growing trees or fast growing provenances did not increase in proportion as tracheid width and tracheid diameter increased. This would lead to the decrease in wood basic density. This result was demonstrated in Table 7 by correlation coefficients of wood density with tracheid anatomical features at different growth stage for loblolly pine provenances. There wood density was significant correlated negatively with tracheid width and tracheid diameter at different growth stage of loblolly pine. In Table 8 wood density for fast growing provenances with larger DBH and larger tree volume was smaller, for example, provenances of No15, No 13,

No 16, No 25, No 10, No 31, No 29 and No 20. As pulpwood the decrease of wood density from fast provenances would be compensated with large tree growth increments and high yield per unit and the provenance selection might not be paid much attention to its wood density. But as construction timber the wood property like wood density should be taken into more consideration. These should be stressed in tree breeding and wood improvement of loblolly pine.

IV. CONCLUSION

Based on the present study, the following conclusions can be drawn:

1 There were four radial variation patterns of wood basic density (BD) within 310 trees of 30 loblolly pine provenances, but the main pattern was a steadily increasing trend from pith to bark. The early-late correlation coefficient of BD for loblolly pine strengthens with trees age. The BD value of 5-year-old loblolly pine is able to predict that in 10 years old trees.

2 The BD mean change of 30 loblolly pine provenances is similar to normal distribution, the BD varies from 0.343 to 0.412 g/cm3. The BD mean of the contrast provenance is 0.369 g/cm3 and in medial level. Statistical difference in BD is found among 30 loblolly pine provenance. Further analyses show that the difference among trees within a provenance is greater than that among provenance and the environment has significant effects on the BD of loblolly pine. The broad heritability of BD for loblolly pine is 0.72. The individual tree selection within a provenance was much better than that among different provenance.

3 Wood basic density (BD) of 30 exotic loblolly pine provenances was related positively to the provenance latitude. Longitude of provenance was related negatively to wood density in East Coast and positive to wood density in South Coast and Gulf. There was a tendency for BD of loblolly pine provenance to be lower from west to east and from north to south, this variation in plantation in a common environment turned out contrary to that of natural at different environment. The BD of loblolly pine provenance introduced from high latitude was greater than that from low latitude, but tree growth properties from low latitude were much faster than those from high latitude.

4 The BD mean of 310 trees at the stage of the same age was negatively related to the diameter at breast height and volume of these trees. Wood density was significant correlated negatively with tracheid width and tracheid diameter at different growth stage of loblolly pine. DBH was significant positively correlated with tracheid width, tracheid diameter and negatively distinct related to ratio of tracheid double wall thickness to tracheid diameter. That DBH was related positively to ratio of tracheid diameter to tracheid width and a little negatively to tracheid wall thickness showed that the tracheid wall thickness of fast trees or fast provenances did not increase in proportion as tracheid width and tracheid diameter increased. This would lead to the decrease in wood basic density. Better provenance selection for pulpwood and building lumber should be determined from both tree growth and wood properties.

ACKNOWLEDGMENTS

This paper was a part work of the project (IFS D/2260) funded by the International Foundation for Science in Sweden and by Natural Scientific Foundation of China (NSFC 30271056). We would like to express my thanks to Prof. Raul M De Albuquerque Sardinha, Prof. Jiang Zehui, Prof Li Minghe for their helpful suggestions in plan design, test methods and this paper writing. The direct great contributions have been made to this paper by Prof. Liu Zhaoxi and my graduates who are Miss Huang-Rui, Mr. Xiang Chunyun.

REFERENCES

[1] Byram T D, and Lowe W J. 1998, Specific gravity variation in a loblolly pine seed source study in the western Gulf region. Forestry Science, 34(3): 798-8032.

[2] Bunn E H. 1981, The nature of the resource. New Zealand Journal of Forstry. 26:162-169.

[3] de Guth E B. 1970, Variation of physical and chemical characteristics of several subtropical pinus species in North Argentina.. IDIA, 6:33-40.

[4] Jackson L W, Strickland R E 1962, Geographic variation in tracheid length and wood density of loblolly. Georige Rec Counc 8:1-4

[5] Jayawickrama K, McKeand S E and Jett J B. 1997, Date of earlywood-latewood transition in provenances and families of loblolly pine, and its relation to growth phenology and juvenile wood specific gravity. Can. J. For. Res. 27:1245-1253.

[6] Jett J B, Guiness W M . 1992, Growth and wood properties in a Carolina sandhills pine seed source study. Southern-Journal-of-Applied-Forestry. 1992, 16: 4, 164-1696.

[7] Lantz V W, and Hofmann J G,1969, Geographic variation in growth and wood quality of Loblolly Pine in North Carolina. Proceedings of the 10th Southern Conference on Forest Tree Improvement. Houston,Texas , p175-188

[8] Liu Qinghua, hang Rui, Jin Guoqing. Variation of Ring Width and Wood Basic Density and Early Selection of Pinus massoniana Provenances[J]. Scientia Silvae Sinicae, 2010, 46(5): 49-54.

[9] Loo C C, Tauer and Mcnew R W. 1985, Genetic variation in the time of transition from juvenile to mature wood in loblolly pine. Silvae Genetica,34(1)14 - 19

[10] Koch P. 1972 Unitilization of southern pines. USDA For. Serv.Agric. Handb.pp1630.

[11] Kimura Junji , Fujimoto Takaaki. Moeling the effects of growth rate on the intra-tree variation in basic density in hinoki cypress..Journal of wood science 60(5), 305-312, 2014

[12] Panshin A J, Zeeuw C. Textbook of wood technology, 4th ed, New York, McGraw-Hill, 1980

[13] Peng Zigan. 1992, Studies on provenance trial of slash pine and loblolly pine(in chinese). First Edition , Beijing Science and Technology Publishing House. China Beijing , 181pp.

[14] SAS Institute Inc. 1988a.SAA user,s guide: basic, version 6.03 edition. SAS Institute Inc., Cary, NC.

[15] S K Sharma,S R Shukla and S. Shashikala. Axial variations in anatomical properties and basic density of Eucalypt urograndis hybrid. J.For.Res.(2015)26(3):739–744

[16] Talbert J T, and Jett J B. 1981, Regional specific gravity values for plantation grown loblolly pine in soutreastern of United States. For. Sci. 27:801-807

[17] McLemore B F, Crow A B and Wakeley, P C. 1961, Dry-matter content of Loblolly Pine needles appears unrelated to geographic seed source. For. Sci. 7 (4), 1961 (373-5).

[18] Takeuchi Ryosuke , Wahyudi Imam , Aiso Haruna.Wood properties related to pulp and paper quality in twoMacaranga species naturally regenerated in secondary forests, Central Kalimantan, Indonesia. Tropics 25(3), 107-115, 2016 (JAPAN SOCIETY OF TROPICAL ECOLOGY)

[19] Tauer C G and Loo Dinkins J A. 1990, Seed source variation in specific gravity of loblolly pine grown in a common environment in Arkansas. Forestry Science, 36(4): 1133-1145

[20] Thor E. 1967, A ten-year-old Loblolly Pine seed source test in Tennessee. J. For. , 65 (5):326-327

[21] Zobel B J. 1958, Natural variation in wood specific gravity of loblolly pine and analysis of contributing factors. Tappi 41(1)14-19

[22] Zobel B J and J P van Buijtenen. 1989, Wood variation, its cause and control. Springer-Verlag.

Effects of Different Media on Micropropagation and Rooting of Myrtle (*Myrtus communis* L.) in *In Vitro* Conditions

Özhan Şimşek[1*], Belgin Biçen[1], Dicle Dönmez[2], Yıldız Aka Kaçar[1,2]

[1]Çukurova University, Agriculture Faculty Horticulture Department, Adana, Turkey
[2]Çukurova University, Institute of Natural and Applied Sciences, Biotechnology Department, Adana, Turkey
*Corresponding author: ozhan12@gmail.com

Abstract— *Myrtle (Myrtus communis L.) is a small tree shrub of the family Myrtace, grown naturally of the Mediterranean area. Myrtle is very important as an antiseptic, anti-inflammatory and hypoglycemic agent. Turkey has great genetic resources for myrtle. Propagation of myrtle genotypes is significant issue. Plant tissue culture techniques offer fast and reliable micropropagation for many plant species. Different media content could be used for micropropagation in in vitro condition. The aim of the present study is to determinate of effects of different media on micropropagation and rooting in myrtle. For this purpose, Murashige and Skoog (MS), Rugini Olive Medium (OM) and Woody Plant Medium (WPM) media were used for micropropagation and rooting experiments. All media were supplemented with 1 mg l^{-1} BA for micropropagation, 1 mg l^{-1} IBA for rooting. The rate of micropropagation and plant length, rooting rate, numbers of root and root length were determined. Rooted with well-developed shoots transferred to plastic pots containing autoclaved peat and perlite (1:1, v/v). The potted plants were placed in a greenhouse. Acclimatized plants were compared after eight weeks. Means were separated by analysis of variance and the LSD test was performed to examine significant differences. Based on the result, the best medium was detected WPM on micropropagation rate (6.75 per plant), and then MS (4.20 per plant), OM (3.70 per plant). According to rooting data the highest rooting rate was calculated in WPM with 100%, rooting rate in OM and MS media was detected 70% and 50%, respectively.*

Key Words— In vitro, plant tissue culture, MS, BA, IBA.

I. INTRODUCTION

Myrtus communis L., commonly named Myrtle, is an aromatic shrub of the Myrtaceae family, widespread all around the Mediterranean basin [1].

Myrtle generally grows in the areas with an altitude of 500–600 m above the sea level, especially in pine forests and riversides in the Taurus Mountains of Turkey. Myrtle is also named as "hambeles", "mersin" or "murt" in Turkish [2]. It can be grown successfully in soils under hot, arid environments. Besides the consumption of its fruits, myrtle, an aromatic woody species, is also utilized for ornamental purposes and for the production of antimicrobial compounds. This species has genotypes with bluish-black or yellowish-white colored fruits [3].

M. communis has a long history of use as food preservative and in traditional medicine [1]. The aromatic biochemical compounds isolated from myrtle are used for anti-genotoxic, anti-mutagenic, antiseptic, anti-inflammatory purposes and culinary purposes, such as for the flavoring of sauces, confectionery, and beverages [4, 5,6,7]

Micropropagation is one of the most important applications of plant tissue culture. It provides numerous advantages over conventional propagation like mass production of true-to-type and disease-free plants of elite species in highly speedy manner irrespective of the season requiring smaller space and tissue source. Therefore, it provides a reliable technique for *in vitro* conservation of various rare, endangered and threatened germplasm. Micropropagation protocols have been standardized for commercial production of many important medicinal and horticultural crops [8]

Myrtle is known as among recalcitrant species. Therefore many researcher study in *in vitro* micropropagation and rooting of myrtle. The effects of indole-3-butyric acid (IBA) and activated charcoal (AC) concentrations for *in vitro* rooting in Myrtle were investigated by Aka Kacar [9]. Şan et al. [6] investigated effects of different concentrations of thidiazuron (TDZ), 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) on shoot formation and the effect of activated charcoal on rooting for myrtle clone 'Aşı Mersin'. Rezaee and Kamali [10] studied on effect of different media (MS and Woody Plant Medium-WPM) of myrtle micropropagation and rooting in *in vitro* conditions. Scarpa et al. [11] evaluated the influence of

two IAA concentrations (0.5 mg l^{-1} and 1 mg l^{-1}) and different medium for root induction in myrtle. Hatzilazarou et al. [12] investigated rooting capability of two *M. communis* clones.

Different plant tissue culture media could be used for routine micropropagation and rooting in *in vitro* conditions. Several species may be different responses on different media content in *in vitro*. The aim of this study is determined of effect of different plant tissue culture media in *in vitro* propagation and rooting on myrtle.

II. MATERIAL AND METHODS

2.1 Plant Material

Myrtle, *Myrtus communis* L., genotype was used for *in vitro* micropropagation and rooting experiments. Plant material was obtained from a commercial seedling company in Adana, Turkey.

2.1.1 Surface Sterilization

Shoot tips were cut from donor plants and washed under tap water about 10 min, dipped in 70% ethanol for 2 min, and then immersed in 10% sodium hypochlorite solution (commercial bleach solution with 4.5 % active chlorine, v/v, NaOCl; Domestos®) with 1–2 drops of Tween-20 for 20 min. afterwards they were washed three times with sterile distilled water.

2.2 Multiplication Medium

MS [13], OM [14] and WPM [15] medium supplemented with 1 mg l^{-1} BAP (benzylaminopurine), 30 g l^{-1} sucrose, 8 g l^{-1} agar, pH 5.8 were prepared and autoclaved at 121 ºC for 15 min. Shoot tips of *M. communis* were cultured and propagated on MS medium, and incubated in a growth chamber at 25±2 ºC under cool white fluorescent light at 16 h photoperiod condition. At the end of the three subculture, micropropagation rate, average plant length (cm), number of leaf, fresh weight (g) and dry weight (g) were recorded.

2.3 Rooting Medium

Shoots were transferred to MS, OM and WPM medium with same components with multiplication medium except BAP. For rooting 1 mg l^{-1} IBA were used. The cultures were maintained incubated in a growth chamber at 25 ºC under cool white fluorescent light at 16 h photoperiod condition. Six weeks later rooting rate, numbers of roots, fresh weight (g), dry weight (g), length of roots and plants (cm) were recorded.

2.4 Acclimatization and *Ex Vitro* Conditions

Plantlets with well-developed shoots and roots were removed from different media boxes and the roots were washed gently under running tap water and dipped in a solution containing 50% (w/v) of a 2.5 g l^{-1} fungicide (Captan 50WP, Fruit&Ornamental, NY, USA) for 10-15 s and then transferred to plastic pots (7 cm × 7 cm width and length) containing autoclaved peat (Klasmann, KTS-1) and perlite (1:1, v/v). The potted plants were placed in a greenhouse under natural light at 95-98% relative humidity and 22-24°C.

2.5 Experimental Design and Statistical Analysis

All experiments were performed in a completely randomized design with three replicates. All quantitative data expressed as percentages were subjected to arcsine transformation. All data were expressed as means. Means were separated by analysis of variance and LSD (the least significant difference) test was performed to evaluate differences among media in myrtle. Data analysis was performed by using the JMP® software (SAS Institute, Cary, NC) ver. 8.00 and significance was considered at $P < 0.05$.

III. RESULTS AND DISCUSSION

3.1 Micropropagation on Media

Micropropagation opportunities of myrtle were investigated using different plant tissue culture media. The rate of micropropagation and plant length, numbers of leaf, fresh weight and dry weight were determined on WPM, MS and OM media supplemented with 1 mg l^{-1} BAP. All data coming from micropropagation studies were presented in Table 1 and Fig.1.

TABLE 1
DATA OF MICROPROPAGATION ON DIFFERENT MEDIA

Medium	Plant Length (cm)	Multiplication Coefficient	Number of Leaf per Plant	Fresh Weight (g)	Dry Weight (g)
WPM	3.40	6.75a	41.30	0.20a	0.038a
MS	3.17	4.20a	34.45	0.14ab	0.019a
OM	2.77	3.70b	31.90	0.07b	0.013b

$LSD_{PlantLength}$=N.S. $LSD_{MultiplicationCoefficient}$= $LSD_{NumberofLeaf}$=N.S. $LSD_{FreshWeight}$=0.79** $LSD_{DryWeight}$=0.01**
N.S.=Not Significant
**=P<0.01

Based on the micropropagation results, WPM was detected to be the best medium on all criteria. Plant length and numbers of leaf was determined that there is no statistically a difference among the media. The highest multiplication coefficient was obtained from WPM with 6.75. MS and OM has lower multiplication coefficient compared to WPM.

Fresh and dry weight of plants on micropropagation was also determined. The best data of mass was obtained from WPM similar to multiplication coefficient.

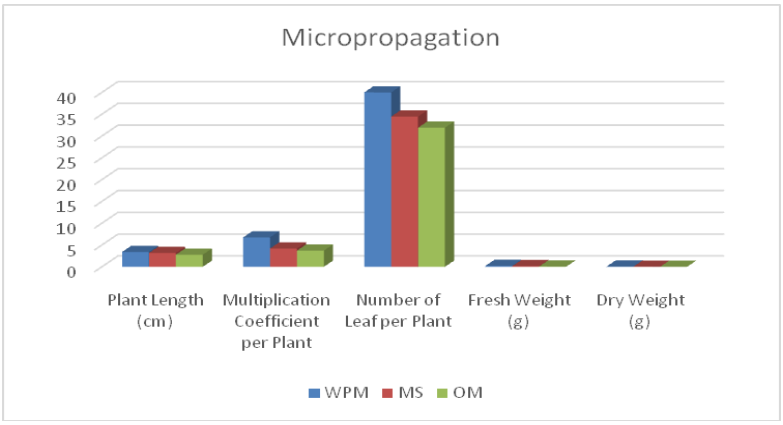

FIG. 1. ALL DATA COMING FROM MICROPROPAGATION STUDIES

Micropropagated myrtle plants on different media were showed in Fig. 2.

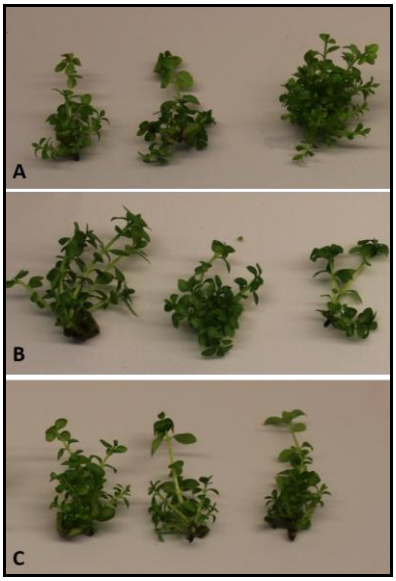

FIG 2. MYRTLE PLANTS A. WPM, B. OM, C. MS

Researchers have been used different nutrient media, plant growth regulators to optimize the micropropagation of myrtle plants in in vitro conditions for many years. Şan et al. [6] investigated the effects of different concentrations of TDZ (0.1, 0.3 and 0.5 mg l^{-1}), BAP (0.5, 1.0 and 2.0 mg l^{-1}) and NAA (0.01 and 0.1 mg l^{-1}) on shoot formation in myrtle clone 'Aşı Mersin'. They used MS medium as micropropagation medium. The most of the shoots were obtained MS medium containing 0.3 mg

l^{-1} TDZ + 0.1 mg l^{-1} NAA in the 1st subculture (3.8 shoot), and from MS with 0.3 mg l^{-1} TDZ + 0.01 mg l^{-1} NAA in the 2nd subculture (4.0 shoot). In our study, the best micropropagation coefficient was calculated as 6.75 in WPM.

Rezaee and Kamali [10] investigated effect of different media and a different plant growth regulations and concentration of shoot proliferation in myrtle. The results indicated that between, MS and WPM, media, WPM showed better result rather than MS. In shoot proliferation stage were used BAP at levels of 0, 0.5, 1, 2, 3, 4, 5 and 6 mg l^{-1} and IBA at levels of 0 and 0.1 mg l^{-1}, then three traits containing: leaf number, shoot length, and shoot proliferation were examined. The best result was obtained by using of modified WPM medium with BAP in 4 and IBA in 0.1 mg l^{-1}. In the present study, we also used 1 mg l^{-1} BAP and we had positive results on multiplication rate.

Damiano et al. [16] carried out propagation studies in myrtle in order to find an efficient propagation protocol. MS medium with BAP (0.6 mg l^{-1}), NAA (0.01 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) was used for propagation of myrtle.

3.2 Rooting on Media

Rooting of myrtle was investigated in in vitro conditions using same plant tissue culture media with micropropagation. The rate of rooting and plant length, root length, numbers of root, numbers of leaf, fresh weight and dry weight were determined on WPM, MS and OM media supplemented with 1 mg l^{-1} IBA. All data coming from in vitro rooting studies were presented in Table 2 and Fig 3.

TABLE 2
DATA OF ROOTING ON DIFFERENT MEDIA

Medium	Rooting Rate (%)	Plant Length (cm)	Root Length (cm)	Number of Root per Plant	Number of Leaf per Plant	Fresh Weight (g)	Dry Weight (g)
WPM	100.00a (90.00)	5.15	5.15a	3.40	15.30	0.16a	0.02a
MS	50.00b (45.00)	5.20	1.00b	0.80	15.80	0.06b	0.01b
OM	70.00ab (72.00)	6.80	4.30a	2.10	16.90	0.11ab	0.02a

$LSD_{RootingRate}$=32.18** $LSD_{PlantLength}$=N.S. $LSD_{RootLenght}$=1.86*** $LSD_{NumberofRoot}$=N.S. $LSD_{NumberofLeaf}$=N.S.
$LSD_{FreshWeight}$=0.06* $LSD_{DryWeight}$=0.01*
N.S.=Not Significant
*P<0.05
**=P<0.01
***=P<0.001

Values in parenthesis arcsine transformed

All plantlets on WPM were rooted. The second best medium was detected to be OM with 70%. The lowest rooting rate (50%) was calculated on MS medium. Comparing plant length among media, there is no any statistically differences. On the other hand, there is significant difference based on the root length. Plantlets coming from WPM has the best value with 5.15 cm per plant. WPM was also the best medium according to numbers of root per plant (3.40). The lowest root number was detected on MS medium with 0.80. Fresh and dry weight of roots on rooting was also determined. The best data of mass was obtained from WPM.

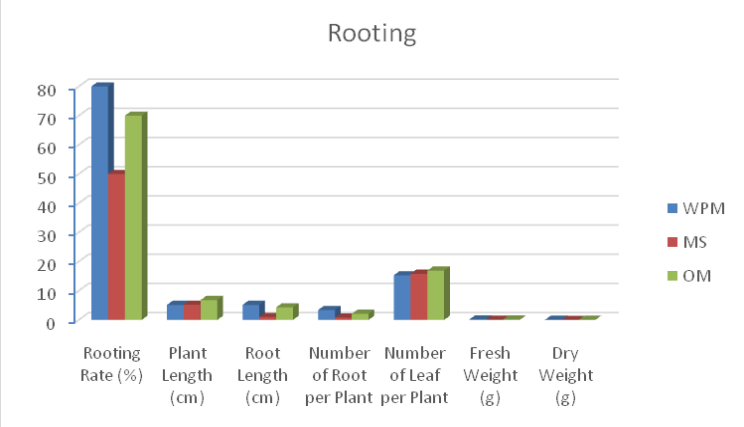

FIG 3. ALL DATA COMING FROM ROOTING STUDIES

Rooted plants on different media were showed in Fig. 4.

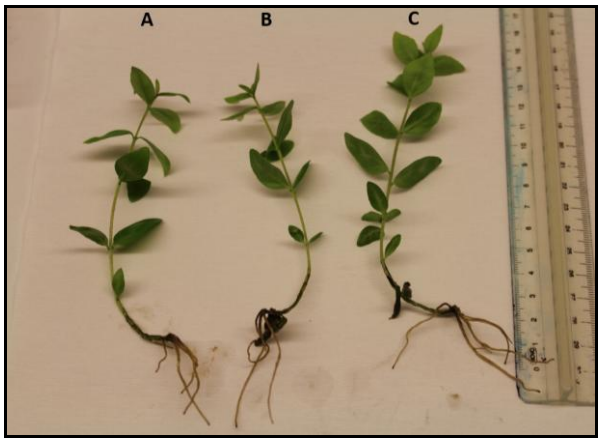

FIG 4. MYRTLE ROOTED PLANTS A. MS, B. OM, C. WPM

Activated Charcoal (AC) has been widely used in tissue culture studies. At the rooting stage, AC delays the disintegration of the auxin added into the medium and positively affects plant development by adsorbing the inhibitors such as phenolic substances and abscisic acid [17]. Şan et al. [6] in order to root the *in vitro* shoots, plantlets were cultured in ½ MS medium containing 0, 0.5, 1.0, 2.0 and 4.0 mg l^{-1} IBA or NAA alone or in combination with 2.0 g^{-1} AC. IBA applications induced more rooting than NAA. The medium supplemented with 1.0 mg l^{-1} IBA + 2.0 g l^{-1} AC resulted in the highest rooting ratio (80%). Addition of AC into the medium resulted in slight increase in the rooting ratio, significant increase in shoot length, and reduced darkening in the rooting area. In our study, high quality roots were obtained from all media. Different auxins can be used for rooting of plants in *in vitro* cultures. Auxins such as NAA, IBA, IAA routinely use for plant rooting in vitro cultures. Scarpa et al. [11] evaluated the influence of two IAA concentrations (0.5 mg l^{-1} and 1 mg l^{-1}) and different medium to induce root induction of Myrtle. Researchers found that the best rooting results (61%) were obtained with O13 medium containing 1 mg l-1 IAA. Hatzilazarou et al. [12] investigated that rooting capability of two M. communis clones, with large (clone A) and small (clone B) leaves. Shoots transferred to WPM medium supplemented with various concentrations (0, 0.5, 1 or 2 µM) of IBA, IAA or NAA in rooting assays. According to this study, the best rooting was achieved with the application of 0.5 µM IBA (96% rooting) and 1 µM IAA (100% rooting) for clone A and B, respectively. Ruffoni et al. [18] determined that IAA and IBA at 0.5 mg l^{-1} increased the rooting percentage and evidenced a difference in the shape and length of the roots in myrtle plant. Aka Kacar et al. [9] investigated the effects of different IBA (0, 1, 2, 4 mg l^{-1}) and AC (0, 0.5, 1, 2 g l^{-1}) concentrations on myrtle rooting in *in vitro* conditions. They used agar MS medium for rooting and reported that the best combination were detected 2 mg l^{-1} IBA without AC in terms of rooting rate.

3.3 Acclimatization

Plantlets with shoots and roots from different media were transferred to plastic pots. Plants were successfully acclimatized in a greenhouse. There were no statistically differences among the treatments on survival. However, plants from WPM showed better performance on survival.

IV. CONCLUSION

Many different biotechnological methods can be applied to plant to have better ones in the process of plant breeding. One of the widest applications of biotechnology has been in the area of plant tissue culture in fruit crops. Today plant tissue culture applications encompass much more than clonal propagation and micropropagation [19].

In conclusion, the WPM, OM and MS medium represent a valid in vitro micropropagation and rooting for the mass propagation. We showed that different media content could be used for myrtle micropropagation and rooting. Comparative investigations among different media that shoot proliferation, rooting and growth were more efficient in WPM.

ACKNOWLEDGEMENTS

This research was supported by Çukurova University, Scientific Research Project Unit (Project No: FBA-2015-4865).

REFERENCES

[1] A. Bouzabata, C. Cabral, M. J. Gonçalves, M. T. Cruz, A. Bighelli, C. Cavaleiro, J. Casanova, F. Tomi, and L. Salgueiro, "*Myrtus communis* L. As Source of A Bioactive and Safe Essential Oil", Food Chem Toxicol, vol. 75, pp. 166-172, 2015.

[2] C. Aydın, and M. M. Özcan, "Determination of Nutritional and Physical Properties of Myrtle (*Myrtus communis* L.) Fruits Growing Wild in Turkey", J Food Eng, vol. 79(2), pp. 453-458, 2007.

[3] B. Şan, A. N. Yildirim, M. Polat, and F. Yildirim, "Chemical Compositions of Myrtle (*Myrtus communis* L.) Genotypes Having Bluish-Black and Yellowish-White Fruits", Erwerbs-obstbau, vol. 57(4), pp. 203-210, 2015a.

[4] G. H. S. Bonjar, "Antibacterial Screening of Plants Used in Iranian Folkloric Medicine", Fitoterapia, vol. 75, pp. 231–235, 2004.

[5] N. Hayder, A. Abdelwahed, S. Kilani, R. Ben Ammar, A. Mahmoud, K. Ghedira, and L. Chekir-Ghedira, "Anti-Genotoxic and Free-Radical Scavenging Activities of Extracts From (Tunisian) *Myrtus communis*", Mutat Res, vol. 564, pp. 89–95, 2004.

[6] B. Şan, Y. Karakurt, and F. Dönmez, "Effects of Thidiazuron and Activated Charcoal on In Vitro Shoot Proliferation and Rooting of Myrtle (*Myrtus communis* L.)", Tarım Bilimleri Dergisi, vol. 21(2), pp. 177-183, 2015b.

[7] S. Kordali, A. Usanmaz, A. Cakir, A. Komaki, and S. Ercisli, "Antifungal and Herbicidal Effects of Fruit Essential Oils of Four *Myrtus communis* Genotypes", Chem Biodivers, vol. 13(1), pp. 77-84, 2016.

[8] A. Shahzad, S. Sharma, S. Parveen, T. Saeed, A. Shaheen, R. Akhtar, V. Yadav, A. Upadhyay, and Z. Ahmad, "Historical Perspective and Basic Principles of Plant Tissue Culture", In Plant Biotechnology: Principles and Applications, Springer Singapore, 2017, pp. 1-36.

[9] Y. Aka Kacar, Ö. Şimşek, B. Biçen, and B. Dal, "*In Vitro* Rooting of Micropropagated Shoots From *Myrtus communis* Linn: Influence of Activated Charcoal and Indole-3-Butyric Acid (IBA)", Acta Hort, 2017. 10.17660/ActaHortic.2017.1155.78.

[10] A. Rezaee, and K. Kamali, "A New Commercial Protocol for Micropropagation of Myrtus tree", Adv Biores, vol. 5(4), pp. 73-79, 2014.

[11] G. M. Scarpa, M. Milia, M. Satta, "The Influence of Growth Regulators on Proliferation and Rooting of *In vitro* Propagated Myrtle", Plant Cell Tiss Org, vol. 62, pp. 175-179, 2000.

[12] S. Hatzilazarou, H. Grammatikos, A. S. Economou, N. Rifaki, and P. Ralli, "Rooting *In Vitro* and Acclimatization of *Myrtus communis* Microcuttings", Acta Hort (ISHS), vol. 616, pp. 259-264, 2001.

[13] T. Murashige, and F. Skoog, "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures", Physiol Plantarum, vol. 15, pp. 473-497, 1962.

[14] E. Rugini, *"In* Vitro Propagation of Some Olive Cultivars with Different Root-Ability and Medium Development Using Analytical Data from Developing Shoots and Embryos", Sci Hortic, vol. 24, pp. 123–134, 1984.

[15] G. B. Lloyd, and B. H. McCown, "In: Proceedings of the International Plant Propagation Society", vol. 30, pp. 421-421, 1980.

[16] C. Damiano, M. D. A. Padró, and A. Frattarelli, "Propagation and Establishment *In Vitro* of Myrtle (*Myrtus communis* L.), Pomegranate (*Punica granatum* L.) and Mulberry (*Morus alba* L.)", Propag Ornam Plants, vol. 8(1), pp. 3-8, 2008.

[17] T. D. Thomas, "The Role of Activated Charcoal in Plant Tissue Culture", Biotechnol Adv, vol. 26(6), pp. 618-631, 2008.

[18] B. Ruffoni, C. Mascarello, and M. Savona, "*In Vitro* Propagation of Ornamental Myrtus (*Myrtus communis*)", In: J. Shri Mohan, O. Sergio J. (Eds.), Protocols for *In Vitro* Propagation of Ornamental Plants Methods in Molecular Biology, 2010, vol. 589, pp. 257-269.

[19] D. Dönmez, Ö. Şimşek, and Y. Aka Kaçar, "Genetic Engineering Techniques in Fruit Science", Int J Environ Agric Res (IJOEAR), vol. 2(12), pp. 115-128, 2016.

AD Publications
Sector-3, MP Nagar, Rajasthan, India
www.adpublications.org, www.ijoear.com, info@ijoear.com