Volume 24 2016 ISSN 1023-8654

JOURNAL OF BIO-SCIENCE

Institute of Biological Sciences
University of Rajshahi, Bangladesh
http://www.banglajol.info/index.php/JBS/index

THE
JOURNAL OF BIO-SCIENCE
(J. Bio-Sci.)

ISSN 1023-8645

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Price of the Journal	SARC countries	Other Countries
Each issue	US$ 25.00 + 5.00 (Postage)	US$ 25.00 + 8.00 (Postage)
Each volume	US$ 50 .00+ 5.00 (Postage)	US$ 25.00 + 8.00 (Postage)

In Bangladesh Tk. 300.00 for one issue and Tk. 500.00 each volume.

Published by the Chief Editor, Journal of Bio-Science, Institute of Biological Sciences, University of Rajshahi, Bangladesh, June 2018.

Printed by: Uttoran Offset Printing Press, Greater Road, Rajshahi- 6000, Bangladesh. Tel: +88-0721-773782

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MA Salam[1], MS Hossain[2], MA Haq[1] and MAK Parvez[3*]

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JOURNAL OF BIO-SCIENCE (J. Bio-Sci.)

CONTENTS

J. Bio-Sci. 24: 1-9, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

EFFECT OF DIFFERENT PRUNING OPERATIONS ON THE INCIDENCE AND SEVERITY OF VARIOUS DISEASES OF TEA PLANT

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Abstract

To analyze the effect of different pruning operation on the incidence and severity of different diseases of tea (*Camellia sinensis*) plant. An experiment was carried out at the Bilashchara Experimental Farm of Bangladesh Tea Research Institute (BTRI), Srimangal. Three places of the sections were selected randomly that received LP (Light Prune), DSK (deep skiff), MSK (medium skiff) and LSK (light skiff) operations. Every bush was critically observed before and after pruning operations and all infected diseases were recorded. Disease severity was expressed as percent disease index (PDI). MSTAT program was used for statistical snalysis. After pruning operation, maximum incidence 33.33% and severity 8.20% of Grey brown blight was found in LSK. Horse hair blight maximum incidence (18%) and maximum severity (6.27%) both were found in LSK. In thread blight maximum incidence was in MSK 22.67% and maximum severity was 7% in LSK. The highest % reduction of branch canker both in incidence and severity was observed in LP section followed by DSK, MSK and LSK. In case of Gall disease maximum incidence and severity both was LSK 24.67% and 7.60%. The highest incidence of black rot was in LSK 41.33% and severity 12.87% was in MSK. From the study, it was recommending that without using any chemicals, only by different pruning operations and proper cleaning can reduce the in incidence and severity of those diseases.

Key words: Diseases, pruning, pruning cycles, tea

Introduction

Tea is most widely popular drinking beverage. Bangladesh is a South-Asian country almost bordered by Indian Territory except for a small strip in the South-East by Mayanmar and the Bay of Bengal in the south. The geographical location of tea growing area is restricted only to some green specks between $21^0 30'$ and $26^0 15'$ north latitude and between $89^0 0'$ and $92^0 41'$ east longitude (Ahmed 2005). Tea cultivation in Bangladesh developed concurrently with the North-east Indian tea during the early part of nineteenth century. Bangladesh tea grows in the three fairly divergent ecological zones namely Surma valley in greater Sylhet, Halda valley in Chittagong and Karatoa valley in Panchagarh districts and most of the tea gardens are situated in Moulavibazar, Sylhet and Chittagong (Ahmed et al. 2015 a,b). Tea ecosystem is a complex agro-ecosystem. It comprises tea, shade trees, green crops, forest etc. The intensive mono culture of a perennial crop like tea over an extensive and contiguous area in apparently isolated ecological zones in Bangladesh has formed virtually a stable ecosystem which provided unlimited opportunity for perpetuation and spread of endemic and introduced diseases (Alam 1999). The architecture of tea plantation, variability of plant types and the systemic interaction of various agro-techniques, intercultural operation etc. imposes a significant impact on development of diseases. The most common foliar diseases of tea in Bangladesh are red rust (*Cephaleuros parasiticus*), horse hair blight (*Marasmius equicrinus*), thread blight (*Marasmius*

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pulcher), black rot (*Corticium invisum*), die back (*Colletotrichum gloeosporioides*), grey blight (*Pestalotia theae*), brown blight (*Colletotrichum camelliae*), blister blight (*Exobasidium vexans*), Macrophoma (*Macrophoma theicola*) and gall (*Fusarium oxysporum*). These fungal diseases are predominant in different tea estates of Bangladesh in different periods of time round the year (Sana 1989). In world tea, about 380 fungal and 1 algal diseases have been recorded. The disease spectrum of Bangladesh tea consists of 1 algal disease, 18 fungal diseases and few epiphytes (Sana 1989). These diseases are hitherto the most common, and may be grouped on the basis of the parts of the plant affected as: 1) foliar diseases, 2) stem diseases, and 3) root diseases. In general, increased plant density will tend to increase leaf surface wetness and leaf surface wetness duration, and so make infection by foliar pathogens more likely (Huber and Gillespie 1992). But, of course, how abiotic stress factors interact to affect plants will be key to understanding climate change effects on plants (Mittler 2006); abiotic stress such as heat and drought may contribute to plant susceptibility to pathogens or it may induce general defense pathways which increase resistance. Singh et al. (2004) reported that trees, which have begun to decline in vigor, yield and size of fruit need pruning to help the restore their condition. Moorman and Roxanne (1999) concluded that comprehensive pruning of branches killed by fungi significantly improves the appearance of Scots pines and dogwoods but does not totally eliminate disease from the trees. The fungi involved can be natural inhabitants on the plants' bark, gaining entry to the plant through wounds or natural openings at any time of the year. Many canker-causing fungi remain latent in the infected tissue until plant resistance responses are suppressed during dormancy or reduced by environmental stress (Schoeneweiss 1975 and 1981, Blodgett and Stanoz 1995, Stanoz et al. 1997). In current investigation it can be concluded the pruning of old and undesirable branches stimulated the growth of new shoots to obtain new fruiting wood. Pruning is an essential cultural operation in tea cultivation that is done to convert the plants into a bushy frame and pruning cycle was defined by Hajra (2001) as the interval of time between two successive prunings. This cultural operation has tremendous influences on the micro climate and disease incidence. Besides, the micro climate change, both in cutting and removing of foliages have manifold effects particularly on the incidence and development of diseases as well as multiplication, perpetuation and dissemination of pathogen. Pruning considered as “at best as necessary evil” (Tubbs 1937) as it both stimulates and control growth. In Bangladesh, four years pruning cycle is practiced in tea gardens those are LP- DSK- MSK- LSK. Longer pruning cycle with light form skiff result is reduction of quality of tea (Ali 1970). In tea plantation, different groups of fungicides like carbendazim, copper oxychloride, copper oxide, copper hydroxide, hexaconazole, propiconazole etc. are used for controlling different tea diseases (Ali and Islam 2014). Pruning is an essential cultural operation of tea cultivation that is done to convert the plants into a bushy frame. This cultural operation has tremendous influences on the micro climate and disease management. Besides, the micro climate change, both in cutting and removing of foliages have manifold effects particularly on removal of diseased parts from the bushes automatically. Primarily it is necessary to know the how much the diseases are reduced both in incidence and severity. With the point of view, this study was undertaken- i) to assess the effect of different pruning operations on the incidence and severity of major tea disease and ii) to determine the suitable pruning operation towards the effective disease management.

Materials and Methods

The experiment was carried out at the Bilashchara Experimental Farm of Bangladesh Tea Research Institute (BTRI), Srimangal, Moulavibazar during October 2012 to February 2013. There were three places of the sections selected randomly that would be received LP operation. From each place 50 bushes were further randomly selected. This procedure was applied for DSK, MSK and LSK operations. Every bush was critically observed and all infected diseases were recorded as per following scale (Islam and Ali 2010). The observations were taken before and after pruning operations.

Grades		Scale
0	:	No infection / no disease
1	:	1-20 % infection
2	:	21-40 % infection
3	:	41-60 % infection
4	:	61-80 % infection
5	:	>80 % infection

Disease severity was expressed as percent disease index (PDI) which was calculated by using following formula (Islam and Ali 2010).

$$\text{Disease Incidence (\%)} = \frac{\text{Number of infected bushes}}{\text{Number of total selected bushes}} \times 100$$

The experimental data were compiled and analysed statistically using MSTAT program. Data were compared following LSD (P = 0.05).

Results

A total of six diseases like, grey blight, horse hair blight, thread blight, gall, branch canker and black rot were recorded during the experiment. Of the six recorded diseases, Grey blight was found statistically (P = 0.05) different in severity for pre pruning operation. Maximum incidence of the disease (82%) was found in MSK operation which was statistically similar to LP and LSK sections. Highest severity of the disease (51.73%) was found in DSK receiving section. Lowest incidence 72% was in DSK and lowest severity was 45.70% was in LP (Table1). After pruning operation, the incidence in LP and DSK section was found statistically identical. Similar case was seen in MSK and LSK sections. In case of severity after pruning operations, DSK and MSK section showed statistically significant result. Lowest incidence was 26.66% at DSK and lowest severity was 4.27% at LP (Table1). The highest reduction both in incidence and severity was observed in LP section followed by DSK, MSK and LSK (Fig.1). Horse hair blight was found statistically (P = 0.05) different in severity for pre pruning operation. Highest incidence of the disease (50.67%) was observed in MSK operation which was statistically similar to LP and DSK operations. Maximum severity of the disease (33.40%) was found in MSK receiving section. Lowest incidence was 42.67 % at LSK and lowest severity was 27.53% at LP. After pruning operation, highest incidence (18.00%) and severity (6.27%) of the disease were found in LSK receiving section. Lowest incidence and severity were 10.00% and 2.40% in LP operation (Table 2). The reduction both in incidence and severity was observed in LP section followed by DSK, MSK and LSK (Fig. 2). In case of Thread blight, maximum incidence (60.66%) was found in LP operation which was statistically similar to DSK and MSK. Maximum severity of the disease (39.33%) found in LP receiving section. After pruning, maximum incidence (22.67%) was found in MSK receiving section. The incidence in LP and DSK section was found statistically identical. Similar case was seen in MSK and LP sections. Highest severity of the disease (7%) found in LSK receiving section (Table 3). On the other hand graphical figure revealed that highest % reduction both in incidence and severity was observed in LP section followed by DSK, MSK and LSK (Fig. 3). Gall was found significantly different in severity for pre pruning operation.

Maximum incidence (86.67%) was found in LSK operation. The incidence in LP, DSK and MSK section was found statistically identical. Maximum severity of the disease (50.27%) found in MSK receiving section. Lowest incidence (62.66%) and severity (38.60%) were observed in DSK and LSK. After pruning maximum incidence of the disease (24.67%) was found in LSK operation (Table 4). Accordingly, the highest reduction both in incidence and severity was observed in LP section followed by DSK, MSK and LSK (Fig. 4). Highest incidence (44.67%) of the branch canker disease was found in LP operation which was statistically similar to MSK and LSK sections. Maximum severity of the disease (41%) was found in LP receiving section. After pruning operations highest incidence of the disease (39.33%) was found in DSK operation and maximum severity (12.20%) was found in LSK receiving section (Table 5). The graphical results (Fig. 5) revealed that highest % reduction both in incidence and severity was observed in LP section followed by DSK, MSK and LSK. Another important disease Black rot was found statistically (P = 0.05) different in both incidence and severity for pre pruning operation. Maximum incidence of the disease (87.67%) was found in LSK operation while highest severity of the disease (49.40%) found in MSK receiving section. After pruning maximum incidence of the disease (41.33%) was found in LSK receiving section. The incidence in LP and DSK section was found statistically identical. Highest severity of the disease (12.87%) recorded in MSK section. Lowest incidence 16.67% in LP and lowest severity 7.13% were observed in DSK (Table 6). Fig. 6 revealed that LP pruning has great impact on the significantly reduction of disease incidence and severity followed by DSK, MSK and LSK.

Table1. Effect of different pruning operations on the incidence and severity of grey blight disease of tea.

Different	Pre pruning		Post pruning	
pruning operations	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
LP	80.67	45.70	27.33	4.27
DSK	72.00	51.73	26.66	6.33
MSK	82.00	46.93	32.67	6.27
LSK	80.67	50.67	33.33	8.20
LSD (0.05)	3.52	0.91	5.81	0.73

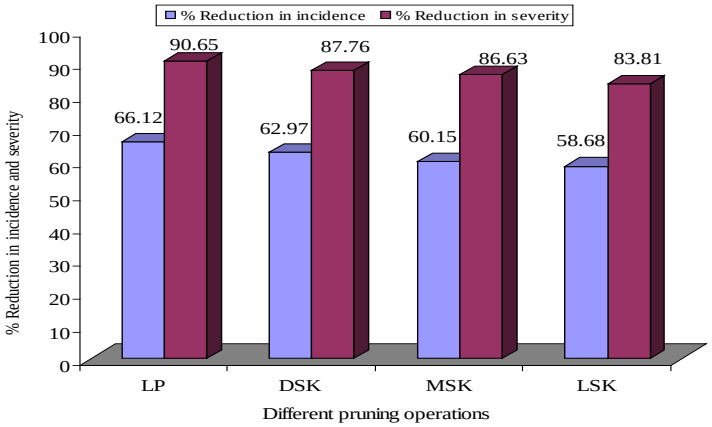

Fig. 1. Showing % reduction in incidence and severity of grey blight due to pruning operation.

Table 2. Effect of different pruning operations on the incidence and severity of horse hair blight disease of tea.

Different pruning operations	Pre pruning		Post pruning	
	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
LP	45.33	27.53	10.00	2.40
DSK	49.33	31.33	13.33	3.13
MSK	50.67	33.40	14.00	5.13
LSK	42.67	27.67	18.00	6.27
LSD (0.05)	5.69	0.94	3.82	0.63

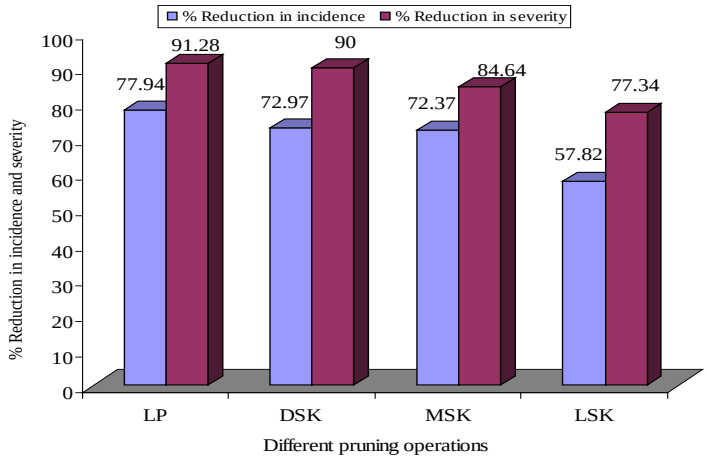

Fig. 2. Showing % reduction in incidence and severity of horse hair blight due to pruning operations.

Table 3. Effect of different pruning operations on the incidence and severity of thread blight disease of tea.

Different pruning operations	Pre pruning		Post pruning	
	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
LP	60.66	39.33	15.33	4.86
DSK	59.33	33.53	17.33	4.27
MSK	54.00	32.26	22.67	5.33
LSK	35.33	29.60	16.67	7.0
LSD (0.05)	6.89	1.12	1.33	0.68
CV	6.59	1.67	3.70	7.78

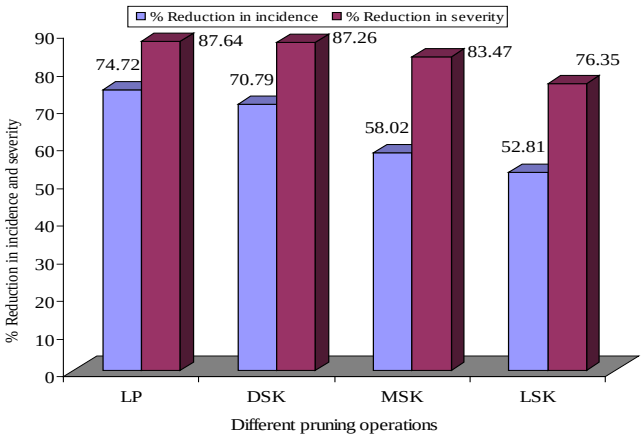

Fig. 3. Showing % reduction in incidence and severity of threat blight disease due to pruning operations.

Table 4. Effect of different pruning operations on the incidence and severity of gall disease of tea.

Different pruning operations	Pre pruning		Post pruning	
	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
	Average of three replications		Average of three replications	
LP	68.67	41.93	14.00	2.87
DSK	62.66	40.80	14.67	4.94
MSK	65.33	50.27	16.00	6.33
LSK	86.67	38.60	24.67	7.60
LSD (0.05)	6.82	1.05	4.80	0.69

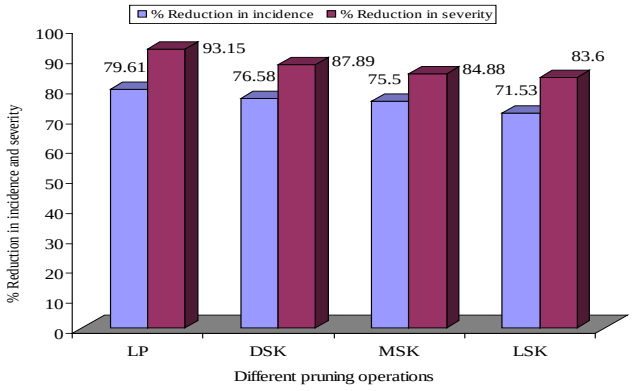

Fig. 4. Showing % reduction in incidence and severity of gall disease due to pruning operations.

Table 5. Effect of different pruning operations on the incidence and severity of branch canker disease of tea.

Different pruning operations	Pre pruning		Post pruning	
	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
	Average of three replications		Average of three replications	
LP	44.67	41.00	27.33	10.07
DSK	28.00	24.87	39.33	8.53
MSK	43.33	26.60	34.00	10.13
LSK	40.00	24.68	35.33	12.20
LSD (0.05)	6.38	0.73	3.05	0.46

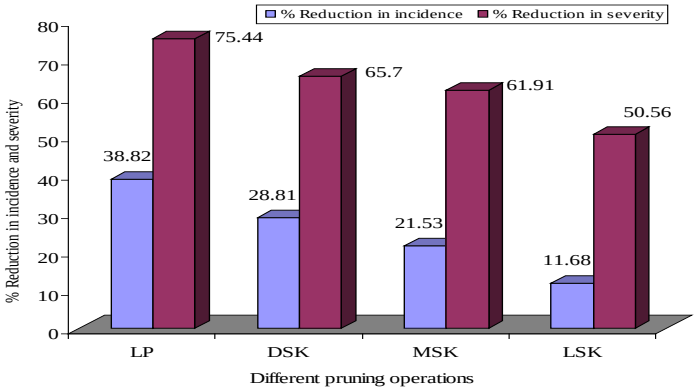

Fig. 5. Showing % reduction in incidence and severity of canker disease due to pruning operations.

Table 5. Effect of different pruning operations on the incidence and severity of Branch canker disease of tea.

Different pruning operations	Pre pruning		Post pruning	
	Incidence (%)	Severity (PDI)	Incidence (%)	Severity (PDI)
	Average of three replications		Average of three replications	
LP	56.00	41.73	16.67	9.80
DSK	51.33	29.20	19.33	7.13
MSK	70.67	49.40	29.33	12.87
LSK	87.67	38.73	41.33	11.67
LSD (0.05)	4.26	1.42	6.82	0.64

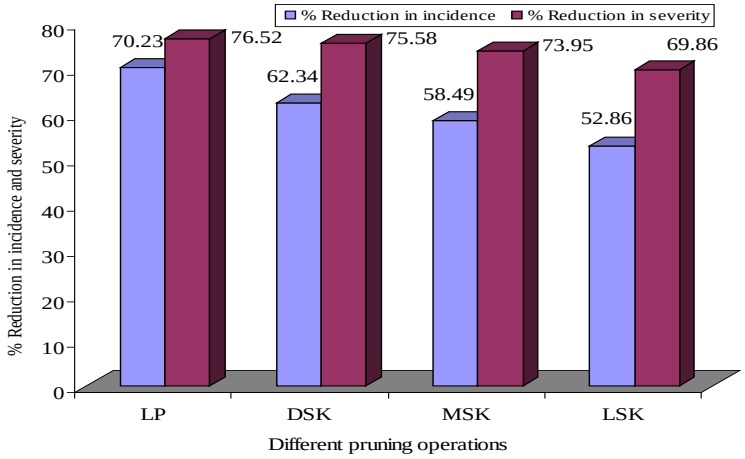

Fig. 6. Showing % reduction in incidence and severity of black rot disease due to pruning operations.

Discussion

Pruning entail targeted removal of diseased, damaged, dead, non productive, structurally unsound, or otherwise unwanted tissue from crop. Luepschen and Rohrbach (1969) demonstrated that wound susceptibility of *Prunus* spp. To *Leucostoma* spp., the pathogen causing a perennial canker disease of stone fruits, varied by time of year and that the application of shellac was beneficial in reducing infection. Similar benefit of pruning pains to control infection of *Malus* spp. With *Cylindrocarpon mali*, another canker disease of apples, have also been demonstrated (Gupta and Agarwala 1972). Biggs (1990) found that wound susceptibility to infection decreases with increasing suberin and lignin formation after wounding. On the other hand Manivel (1980) state that the top layer of maintenance foliage makes the maximum contribution towards the growth of new shouts and as well as reduce the diseases of tea plant. Islam and Ali (2010) observed that the incidence of branch canker to be highest in LP and DSK receiving sections followed by horse hair blight. The incidence of Thread blight disease was found to be comparatively high both in LP and DSK areas. In MSK and LSK receiving sections, Horse hair blight was found to be highest in terms of disease incidence. Similar observation was observed by Uddin et al. (2005) gall disease incidence was low in LP and DSK receiving sections and highest in LSK receiving sections. From the study, it is recommend that without using any chemical only with different pruning operation and proper cleaning can reduce the in incidence and severity of those diseases is possible.

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J. Bio-Sci. 24: 11-18, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

PHYTOCHEMICAL SCREENING OF *SYZYGIUM CUMINI* (L.) EXTRACTS IN DIFFERENT SOLVENTS

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Abstract

Phytochemical screening of the secondary metabolites was performed with acetone, chloroform, methanol and n-hexane extracts of the leaves, roots, stem bark and seeds of the plant *Syzygium cumini* (Linn.) and were detected using various tests for identifying the isolated components. Acetone extract of the leaves showed the presence glycosides, phenols, proteins, resins and saponins while the stem bark extract showed the presence of alkaloids, flavonoids, glycosides, phenols, proteins, resins and saponins. All the above constituents except saponins were detected in the root extract. The seed extract contains alkaloids, carbohydrates, phenols, proteins and tannins. Chloroform extract of the leaves showed the presence of alkaloids, proteins and steroids while root extract contains alkaloids and steroids and that of seed extract alkaloids, carbohydrates, phenols, proteins and tannins, however, alkaloids and tannins were found in the stem bark extract. Methanol extract of the leaves and stem bark showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenols, resins, saponins, steroids and tannins while the root extracts contain all the above constituents along with proteins. N-hexane extract of the leaves contain only alkaloids, roots contain alkaloids and resins and that of seeds contain carbohydrates and proteins while alkaloids, proteins and tannins were found in stem bark extract. The present findings reveal the presence of various medicinally important phytochemicals from the plant *S. cumini* extracts may have application in traditional system of medicine to cure various ailments.

Key words: Extracts, phytochemical, solvents, *Syzygium cumini*

Introduction

Plants are the chemical factories of nature, producing many chemicals, some of which have medicinal and pesticidal properties. Recently, in different parts of the world, attention has been paid towards exploitation of higher plant products as novel chemotherapeutants. The plant components are non phytotoxicity, systemicity, easy biodegradability and stimulatory in nature. Plant products possess the potentials used in pest management (Dubey et al. 2008). Phytochemicals are naturally occurring in the medicinal plants that have defense mechanism and protect the plant from various diseases (Krishnaiah et al. 2007). The non-nutritive plant chemicals have protective or disease preventive properties. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Sibanda and Okoh 2007). The term qualitative phytochemical analysis refers to the procedures involved in establishing and proving the identity of the phytochemical constituents present in the crude plant extract. The pharmacological actions of crude drugs are determined by the nature of their constituents present there in the phytochemicals.

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Syzygium cumini L. is a very common, large evergreen tree of the Indian Subcontinent commonly known as Jamun, belongs to the Myrtaceae family. The original home of *S. cumini* is India or the East Indies. It is found in Thailand, Philippines, Madagascar, West Indies, East and West Africa and some subtropical regions including Florida, California, Algeria and Israel.

The major phytoconstituents are reported to contain vitamin C, gallic acid, tannins, anthocyanins, cyanidin, petunidin, malvidinglucoside and other components (Annonymous 1976, Martinez and Valle 1981). Preliminary phytochemical analysis also showed the presence of phenols, terpenoids, tannins, saponins, phytosterols, carbohydrates, flavonoids, amino acids in the stem bark of *S. cumini* (Kuncha et al. 2012). The stem bark of *S. cumini* also contains butulinic acid, *ß*-sitosterol, friedelin, epi-friedelanol (Annonymous 1976). It also contains new esters of epifriedelanol (eugenin), D-glucoside, kaempterol-3-O-glucoside, quercetin, myricetin, astragalin and gallic acid (Bhargava et al. 1974).

The plant derived compounds have been utilized by the humans from time immemorial in different sectors of life, including public health and pest management. The test plant is also a native to Bangladesh and is easily available. The seeds of *S. cumini* are used as astringent and diuretic (Bhatia and Bajaj 1975). They have hypoglycaemic (Chopra et al. 1958, Mahapatra et al.1985, Stanely et al. 1998a), anti-inflammatory (Chaudhari et al. 1990), antipyretic (Ghosh et al. 1985), psychopharmacological (Chakraborty et al. 1985), hypolipidaemic (Stanely and Menon 1997), and antioxidant (Stanely and Menon 1998b, Banerjee et al. 2005, Bushra et al. 2007) activities. Hence, the present study was made to investigate the phytochemical screening of the *S. cumini* leaves, root, seeds and stem bark extracted in different solvents.

Materials and Methods

Plant collection and identification

The leaves, roots, seed and stem bark of *S. cumini* were collected in fresh condition during the season of maturity June-July, 2011 from Rajshahi University Campus and identified by taxonomical section, Department of Botany, University of Rajshahi. A voucher specimen has been deposited in the herbarium for future reference.

Preparation of plant extracts

The plant materials were separately powdered in a grinder machine. The powdered materials were weighed and placed in separate conical flasks to add sufficient amount of chloroform (500 g × 1500 ml × 3 times followed by filtration through Whatman 1 filter paper at interval of 24 h for 3 times in the same collection flask) to yield the first extracts of the leaves, roots, seeds and stem bark separately. The output extracts were poured in to glass vials and reserved in a refrigerator at 4ºC with proper labeling. For each of the samples four solvents, acetone, chloroform, methanol and n-hexane were used successively.

Preparation of stock solution

A piece of clean sodium is placed in a fusion tube. The lower part of the tube is heated until the sodium melts down. Then a few milligrams of a particular extract is added and heated until the bottom of the tube become dull red. It was dropped into a small mortar containing 15 ml distilled water. The tube broken up with a pestle and the solution was then filtered and the filtrate was used as the stock solution. The stock solution was then used for the specific test described below:

Test of alkaloids (Mayer's test)

Mercuric chloride (1.36 gm) was dissolved in 60 ml and 5 gm of potassium iodide was dissolved in 10 ml of distilled water respectively. These two preparations were mixed and diluted to 100 ml using distilled water. 1 ml of acidic aqueous solution of samples and few drops of reagent was added. Formation of white or pale precipitate showed the presence of alkaloids.

Test of carbohydrates (Molisch's test)

In a test tube containing 2 ml of aqueous extract of the samples, 2 drops of freshly prepared 20% alcoholic solution of alpha-naphthol and mixed, poured 2 ml of concentrated sulfuric acid so as to form a layer below the mixture. Appearance of produce a red violet ring which disappears on the addition of an excess of alkali solution indicated the presence of carbohydrates.

Test of flavonoids

In a test tube containing 0.5 ml of alcoholic extract of the samples, 5 to 10 drops of diluted HCl and small amount of Zn or Mg were added and the solution was boiled for few minutes. Appearance of reddish pink or dirty brown color indicated the presence of flavonoids.

Test of glycosides

A small amount of extracts were dissolved in 1ml water and then aqueous sodium hydroxide was added. Formation of a yellow color indicated the presence of glycosides.

Test of steroids (Salkowski's test)

About 100 mg of dried extract was dissolved in 2 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown color at the interface was an indicative of the presence of steroidal ring.

Test of saponins

A drop of sodium bicarbonate was added in a test tube containing about 50 ml of an extract. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of saponins.

Test of resins

Two ml of chloroform or ethanolic extract was added in 5 to 10 ml of acetic anhydrite and dissolved by gentle heating after cooling, 0.5 ml of H_2SO_4 was added. Bright purple color was produced to indicate the presence of resins.

Test of phenols (Ferric chloride test)

One ml of alcoholic solution of extract, 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green color indicated the presence of phenols.

Test of tannins (Lead acetate test)

A few drops of 1% solution of lead acetate was added. In a test tube containing about 5 ml of an aqueous extract. Formation of a yellow or red precipitate indicate the presence of tannins.

Test of proteins (Biuret's test)

One 1 ml of hot extract was added 5-8 drops of 10% (W/V) copper sulphate solution in a test tube. A red or violet color indicated the presence of protein.

Results

The phytochemical screening was performed with the acetone, chloroform, methanol and n-hexane extracts of the leaves of *S. cumini*. Glycosides, phenols, proteins, steroids and tannins were detected in the acetone extract; alkaloids, proteins and steroids from chloroform extract; alkaloids, carbohydrates, flavonoids, glycosides, phenols, resins, saponins; and tannins from the methanol extract; and only the alkaloids were detected from the n-hexane extract (Table 1).

Table 1. Phytochemical screening of *S. cumini* leaf extracts.

Phytoconstituents	n-Hexane	Acetone	Chloroform	Methanol
Alkaloids	+	+	+	+
Carbohydrates	-	-	-	+
Flavonoids	-	-	-	+
Glycosides	-	+	-	+
Phenols	-	+	-	+
Proteins	-	+	+	-
Resins	-	-	-	+
Saponins	-	-	-	+
Steroids	-	+	+	+
Tannins	-	+	-	+

'+' = Presence; '-'= Absence

Preliminary phytochemical analysis of the stem bark of *S. cumini* showed the presence of flavonoids, glycosides, phenol, proteins, resins and saponins in the acetone extract; alkaloids, carbohydrates, flavonoids, glycosides, phenols, resins, saponins, steroids and tannins were recorded in the methanol extract; only alkaloids and tannins were detected in the chloroform extract wheras alkaloids, proteins and tannins were detected in the n-hexane extract (Table 2).

Table 2. Phytochemical screening of *S. cumini* stem bark extracts.

Phyto-constituents	n-Hexane	Acetone	Chloroform	Methanol
Alkaloids	+	+	+	+
Carbohydrates	-	-	-	+
Flavonoids	-	+	-	+
Glycosides	-	+	-	+
Phenols	-	+	-	+
Proteins	+	+	-	-
Resins	-	+	-	+
Saponins	-	+	-	+
Steroids	-	-	-	+
Tannins	+	-	+	+

'+' = Presence; '-'= Absence

In the root extract all the tested phytoconstituents were detected in the methanol extract; alkaloids, carbohydrates, phenol, proteins and tannins were detected in the acetone and chloroform extracts, but only carbohydrates and proteins were detected from n-hexane extract (Table 3).

Table 3. Phytochemical screening of *S. cumini* root extracts.

Phyto-constituents	n-Hexane	Acetone	Chloroform	Methanol
Alkaloids	+	+	+	+
Carbohydrates	-	+	-	+
Flavonoids	-	+	-	+
Glycosides	-	+	-	+
Phenols	-	+	-	+
Proteins	-	+	-	+
Resins	+	+	-	+
Saponins	-	-	-	+
Steroids	-	+	+	+
Tannins	-	+	-	+

'+' = Presence; '-'= Absence

Screening of seed extracts of *S. cumini* showed that alkaloids, flavonoids, phenols, proteins, resins and tannins were detected in methanol extract; alkaloids, carbohydrates, phenols, proteins and tannins were found in the acetone and chloroform extracts whereas only carbohydrates and proteins were detected in the n-hexane extracts (Table 4).

Table 4. Phytochemical screening of *S. cumini* seed extracts.

Phyto-constitutents	n-Hexane	Acetone	Chloroform	Methanol
Alkaloids	-	+	+	+
Carbohydrates	+	+	+	-
Flavonoids	-	-	-	+
Glycosides	-	-	-	-
Phenols	-	+	+	+
Proteins	+	+	+	+
Resins	-	-	-	+
Saponins	-	-	-	-
Steroids	-	-	-	-
Tannins	-	+	+	+

'+' = Presence; '-'= Absence

Discussion

The extracts of *S. cumini* in different solvents revealed the presence of phytochemicals such as alkaloids, carbohydrates, flavonoids, glycosides, phenol, resins, saponins, tannins and steroids which are summarized in Tables 1-4. The phytochemicals detected are known to have beneficial importance in medical sciences. Alkaloids are the secondary metabolites of plants. They are internal constituents of plants so called as biomolecules. Chemically they contain nitrogen and alkaline in nature. They are found in plant organs like leaves, stem, bark, roots, etc. They are used as medicines in serious disorders like heart-failure, cancer, blood pressure, etc., Euphoric and addicting drugs and pesticides or insect repellents (Nobori et al. 1994). The carbohydrates produced by plants are important source of energy transportation for animals. It is a biomolecules. Carbohydrate derivatives are involved in reproduction, the immune system, the development of disease and blood clotting. Flavonoides are a group of plant metabolites thought to provide health benefits through cell signaling pathways and antioxidant effects and used reduced risk of cancer, heart disease, asthma and stroke (Aiyelaagbe and Osamudiamen 2009). Glycosides is also a molecule and used as atrial flutter, atrial fibrillation, paroxysmal tachycardia, congestive heart failure (Nyarko and Addy 1990).

The total polyphenol contents in methanolic and aqueous fractions of *S. cumini* leaves were found to be 180 and 94 mg/g, respectively, using the Folin-Ciocalteu and acid gallic as standard (Kaneria et al. 2009). An infusion made from the skin of the ripe fruits of *S. cumini* showed the presence of around 100 mg/g of phenolics (Banerjee et al. 2005). Many parts of *S. cumini* including seeds have significant polyphenolic content and the antioxidant properties of these compounds are frequently associated with the antidiabetic effect of many plant species (Sabu et al. 2002, Hanamura et al. 2005).

The resin acts as a bandage protecting the plant from invading insects and pathogens. Plant resins are valued for the production of varnishes, adhesives, and food glozing agents. Saponin seems to reduce blood cholesterol levels, reduce the risk of cancer and stimulate immune system. It is also known to have antifungal properties (Haslem 1989). Ukoha et al. (2011) found tannins bind to proline rice protein and interfere with protein synthesis. It also exhibit antiviral, antibacterial and anti-tumor activities. Steroids are anti-inflammatory, immune-modulating (William et al. 2014). The present findings indicate that phytochemical compounds are the bioactive constituents and this plant is proving to be a valuable reservoir of bioactive compounds of potential health benefits.

Acknowledgement

This research work has been done with the financial assistance of the fund approved as project (Bio-12/2016) by the University Grants Commission of Bangladesh.

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J. Bio-Sci. 24: 19-32, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

EVALUATION OF SOMACLONAL VARIANTS UNDER FIELD CONDITION FOR THE VARIETAL IMPROVEMENT OF STRAWBERRY (*FRAGARIA × ANANASSA* DUCH.) IN BANGLADESH

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Abstract

The present study was conducted to standardize a suitable protocol for varietal improvement of strawberry through somaclonal variation using *in vitro* techniques. Leaf segments of seven strawberry varieties *viz*. AOG, JP-2. JP-3, Camarosa, Sweet Charly, Giant Mountain and Festival were used for callus induction and shoot regeneration. Regenerated plantlets were planted into the field to evaluate their morphological characters, yield contributing characters, response to fungal diseases and summer overcoming potentiality (%) of the transplanted plantlets compare with their parents and data were recorded. Among the seven strawberry varieties AOG was found to be the most responsive genotype for callus induction, shoot regeneration and rooting. A total of 40-45 somaclonal variants from each of the tested varieties were established and maintained in the field and were considered as R_0 plants. There were no somaclones found resistance to fungal diseases but someone's, specially AOG SC 3 showed better tolerance than the donor plants. Comparing with donor plants and other somaclones AOG SC 3 was found better summer overcoming potentiality. It can be acceptable commercially if the good characters exhibited are transmitted through generations or could be used in future breeding programme for the improvement of strawberry varieties in Bangladesh.

Key words: Abiotic and biotic stress, callus induction, somaclonal variation, strawberry

Introduction

Strawberry cultivars are progressively studied and cultivated for its great amount of useful phytonutrients including antioxidants and phytochemicals which are advantageous in reducing the risk of tumorigenesis and heart diseases (Abbas et al. 2017). Strawberry is popular among growers of Bangladesh who get high return on their investments due to its short growing season. There are two main types of strawberry cultivars: short day or June bearing and ever bearing. Temperature may interact with photoperiods in all types of strawberries. Basically, cool temperatures promote and hot temperatures inhibit flowering (Rieger 2006). Short- day cultivars are highly sensitive to temperature. Climatic conditions of Bangladesh in winter, specifically from November to March, seem to be suitable for commercial cultivation of strawberry. There are many strawberry genotypes grown in tropical and sub-tropical environment but fruit of these genotypes are mostly unpalatable (Karim et al. 2015). Recent media reports showed that imported plants were grown well producing flowers and fruits up to March in Bangladesh. During summer and humid rainy season, almost all of the imported plants are perished due to different diseases. Plant tissue culture tools have been used to improve the accessibility of existing germplasm and to create new variations for crop improvement through

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micro-propagation, anther culture, *in vitro* selection, embryo rescue, somaclonal variation, somatic hybridization and genetic transformation. Plants regenerated from calli exhibits great genetic variability in agronomic traits that known as somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variations can enlarged the possibility to create genetic variation in crop plant; mainly plant characters can be altered including plant height, yield, number of flowers/plant, early flowering, grain quality, resistance to diseases, insect and pests, cold, drought and salt (Jain et al. 1998, Patnaik et al. 1999). It has been observed that strawberry cultivation in Bangladesh is highly affected with different diseases and environmental factors (Hossain 2007). Among the different diseases verticillium wilt and crown rot were found to be very prominent and high temperature (above 35°C) affects strawberry cultivation in Bangladesh. In the present study, we focused on the varietal improvement of strawberry through somaclonal variation using *in vitro* techniques and evaluate different morphological, yield contributing characters, fungal disease incidence and summer overcoming capacity of the resulted variants.

Materials and Methods

Plant materials and explants collection

The experiment was carried out at the Plant Breeding and Gene Engineering Laboratory, Department of Botany, University of Rajshahi, Bangladesh in the year 2012. *In vitro* grown leaves of strawberry were used as experimental material for callus induction. Leaf segments were collected from *in vitro* grown plantlets maintained in mentioned Laboratory. Three Japanese (AOG, JP-2 and JP-3) and four North American varieties (Camarosa, Sweet Charly, Giant Mountain and Festival) were tested.

Surface sterilization of explants

Explants were surface sterilized with the help of savlon (ACI Pharma, Bangladesh), Tween-80 and 0.1% Mercuric chloride ($HgCl_2$). To ensure aseptic condition under *in vitro*, all instruments, glassware's and culture media were sterilized by autoclaving with 15 lbs/sq. inch (1.16 kg/cm^2) pressure at 121°C temperature for 20 minutes.

Placement of explants on culture media for callus induction

Sterilized leaves put onto the semisolid MS (Murashige and Skoog 1962) media with different concentrations of NAA (α-napthalene acetic acid) and 2,4-D (2,4-dichlorophenoxy acetic acid) alone or in combination with BA (6-Benzyladenine). The pH of all media were adjusted to 5.7 before addition of agar and sterilized by autoclaving. The culture vials containing explants were placed under dark condition in a room with controlled environment (temperature 25 ± 2°C and humidity 50% and 16 h light/day by white florescent tube lights).

Callus culture and shoot regeneration

After the induction phase, the largest callus initiated from the leaves was sub cultured on to same medium. During callus culture, percentage (%) of explants induced callus, the degree of callus development, the callus colour and nature were recorded. Then the selected calli were placed on medium supplemented with various concentration and combinations of PGRs for shoot regeneration. The percentage of calli producing shoots and total number of shoots/callus were counted in each treatment. The shoots from selected calli were excised and transferred to multiplication medium (MS +1.5 mg/l BA + 0.5 mg/l KIN) for further growth (Ara et al. 2013).

Rooting and acclimatization

When the regenerated shoot apices reached 4-5 cm in length with 5-6 well developed leaves, they were rescued from the culture vessels and separated from each other and cultured individually in test tubes containing 15-20 ml MS and ½MS with or without different combinations of auxins for root induction. Rooted plantlets were gradually acclimatized and were successfully established in the field. Prior to transfer to the field, the culture tube caps were removed and open culture vessels were kept inside the growth chamber. Then, they were taken out from the controlled environment and kept in room temperature to bring them in contact with the normal temperature for acclimatization. After that, a total of 600 plantlets were brought out of the culture vessels carefully and washed thoroughly under running tap water to make it agar gel free then transferred to plastic pots and kept under shady place and covered with polythene sheet to maintain high humidity around the juvenile plants. Finally they were transferred to the field.

Field evaluation and data analysis of variants

Data on eight morphological characters (plant height, number of leaves/ plant, petiole length, number of nodes/stolon, stolon length in cm, number of crowns/plant, canopy size) and eight fruit yield and yield contributing characters (days to flowering, number of flower clusters/plant, number of flowers/ plant, number of fruits/plant, days to fruit harvest, average fruit weight (g), fruit weight/plant (g) were recorded after 1, 2 and 4 months of transplantation. The wide ranges of variations were recorded. A total of 40-45 somaclones (SC) from each of the tested varieties were established and maintained in the field and were considered as Ro plants. From R_0 plants four somaclones from AOG and two from other six varieties were selected. Data were collected from 10 randomly selected plants and different morphological and agronomical characters were recorded and different statistical analysis (Mean ± SE, Analysis of variance, LSD and CV%) were done. Identification of the diseases found in the field was conducted by Plant Pathology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Rajshahi, Bangladesh. Summer overcoming potentiality (%) was measured when naturally temperature raised more than 35°C. Tolerant to fungal diseases and summer overcoming potentiality (%) of the transplanted plantlets were recorded.

Results and Discussion

Callus induction and shoot regeneration

Callus induction can be controlled by the level of plant growth regulators (auxin and cytokinins) in the culture medium. Leaf segments from the *in vitro* grown plants were used to induce callus supplemented with either 2, 4-D or NAA alone or in combination with BA. The cultured leaf explants were induced to develop callus in most of media formulations but degree of effectiveness of callusing of these formulations were different. Among the different PGR formulations, MS medium supplemented with 0.5 mg/l NAA with 1.5 mg/l BA was found to be the most effective media formulation in terms of percentage (%) of explants induced to develop callus and degree of callus development. The media with 2, 4-D and NAA alone at 2.0 - 2.5 mg/l were also found effective PGR formulation for callus development from different parts of strawberry plants. To generate somaclonal variability, induction, maintenance and regeneration of calli are prerequisites because of various abnormalities that occur in genetic constituent during callus culture in artificial conditions are ultimately exhibited in the regenerated plants (Larkin and Scowcroft 1981, Nasrin et al. 2003, Karim et al. 2015). It has been observed in the previous studies that leaf tissue has been studied and shown to have the greatest regeneration capacity of strawberry plant tissues (Jones et al. 1988, Liu and Sanford 1988, Nehra and Stushnoff 1989, Nehra et al. 1990, Jelenkovic et al. 1991, Popescu et al. 1997, Passey et al. 2003). In addition, leaf derived callus produces more shoots than node and root (Popescu et al. 1997). In this investigation, the calli developed from leaf segments in different culture media formulations were sub-

cultured on to regeneration medium (MS formulation) supplemented with different concentration and combination of BA and NAA and the cultures were incubated in light (16 h). Among the different combinations, the highest response to shoot regeneration was noticed in media contained 1.5 mg/l BA and 0.5 mg/l NAA (Table 1 and Fig. 1, D-E).

Table 1. Effect of different concentrations and combination of 2,4-D, NAA and BA in MS medium on callus induction and shoot regeneration from *in vitro* grown leaf explants. Data were recorded after four weeks incubation in dark for callus induction and five weeks of subculture for shoot regeneration.

Growth regulators (mg/l)	Callus induction													
	% of explants induced callus							Adventitious shoot formation						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
2,4-D														
1.0	30	30	30	30	30	30	30	-	-	-	-	-	-	-
2.0	90	81	81	85	85	82	83	-	-	-	-	-	-	-
2.5	60	60	60	60	70	60	60	-	-	-	-	-	-	-
NAA														
1.0	40	30	30	35	40	35	35	-	-	-	-	-	-	-
2.0	90	86	86	90	90	90	90	-	-	-	-	-	-	-
2.5	70	70	70	70	70	75	65	-	-	-	-	-	-	-
NAA + BA														
0.5+0.5	30	10	30	20	20	30	20	-	-	-	-	-	-	-
0.5+1.0	40	30	30	30	20	30	20	-	-	-	-	-	-	-
1.0+0.5	55	55	55	40	40	40	40	-	-	-	-	-	-	-
1.0+1.0	60	50	50	50	50	50	40	-	-	-	-	-	-	-
0.5+1.5	90	90	90	90	90	90	90	3	-	2	2	2	2	2
2,4-D + BA														
3.0+1.0	90	90	90	90	90	90	90	-	-	-	-	-	-	-
3.0+1.5	70	80	80	80	80	80	80	-	-	-	-	-	-	-
4.0+1.0	90	80	80	80	80	80	80	-	-	-	-	-	-	-
4.0+1.5	80	80	80	80	80	80	80	-	-	-	-	-	-	-
5.0+1.0	70	50	50	50	50	50	50	-	-	-	-	-	-	-

Table 1 Contd.

PGR supplements in callus induction medium (mg/l)	BA supplements in shoot regeneration medium (mg/l)	Shoot regeneration													
		Morphological response after 5 weeks of subculture													
		Percentage of calli induced shoot regeneration							No. of multiple shoots/callus						
		1	2	3	4	5	6	7	1	2	3	4	5	6	7
2,4-D	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(2.0)	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NAA	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(2.0)	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4-D + BA	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(3.0 + 1.0)	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NAA + BA	0.1	8	6	6	5	5	4	5	4	2	2	1	1	1	1
(0.5 + 1.5)	0.5	9	4	4	4	3	3	3	4	1	1	1	1	1	1
	1.0	5	2	2	-	-	-	-	1	1	1	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = No response, 1 = AOG, 2 = JP-2, 3 = JP-3, 4 = Camarosa, 5 = Sweet Charly, 6 = Giant Mountain and 7 = Festival

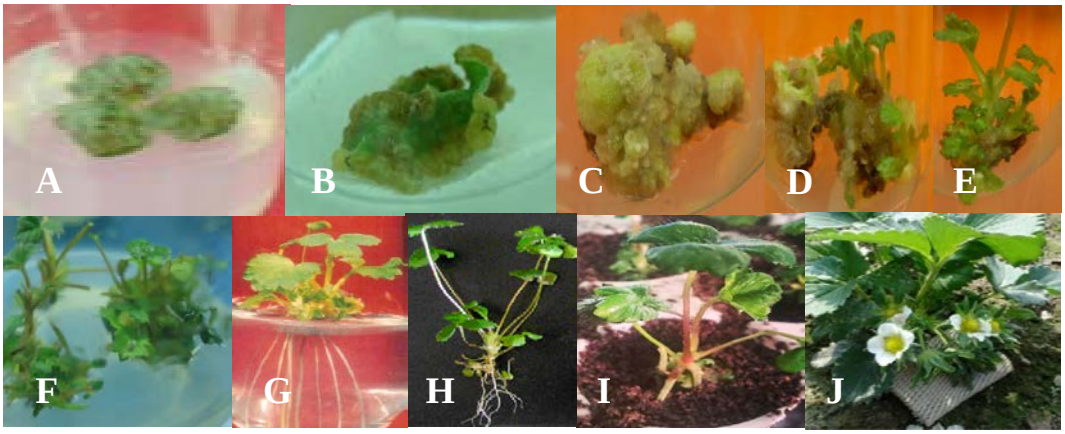

Fig. 1. Initiation of callus from *in vitro* grown leaf after 20 days (A-B) and callus developed after 35 days after culture in MS supplemented with 1.5 mg/l BA + 0.5 mg/l NAA (C). Multiple shoots regenerated in MS medium supplemented with 0.5 mg/l BA which developed in 0.5 mg/l NAA + 1.5 mg/l BA (D-E). Shoot multiplication (F) and rooting (G-H) of regenerated plantlets from leaf derived calli of strawberry. Acclimatization and field establishment of rooted plantlets in plastic pots (I) and in the field with flowers (J).

The kind of PGR and the amount used is varied as the protocols for regeneration of strawberry. Nehra and Stushnoff (1989) were successful with IAA and BA, while six years later, Finstad and Martin (1995) touted the success of 2,4-D and BA. Jelenkovic et al. (1991) studied different cultivars than Nehra or Finstad, tested hypocotyls, runners, petioles, and lamina. Only young fully expanded leaves were used in the lamina study. They determined in preliminary tests that BA and 2, 4-D were the most effective PGR to use. Various combinations of BA, IBA, 2,4-D, KIN, NAA, TDZ, CH, GA_3 and KNO_3 have all been reportedly used in callus induction and plant regeneration studies in strawberry (Liu and Sanford 1988, Nehra et al. 1990; Goffreda et al. 1995, Mahmoud and Kosar 2013, Rahman et al. 2015). Liu and Sanford (1988) reported using casein hydrolysate (CH) and potassium nitrate on leaf explants of 'Allstar' strawberry. Both stimulated the production of callus and shoot and showed additive effect.

Rooting and acclimatization

The micro-shoots of strawberry inoculated in MS and ½MS media and plant growth regulators were induced to develop root without developing any callus at their base. When cultured in MS rooting medium without PGR, all cultured shoots developed roots within 10-15 days of inoculation, whereas 100% of the shoots induced to develop root in MS rooting medium without PGR (Table 2). Addition of auxin in rooting media accentuated rooting, but also microcuttings developed callus at their base, which hampered their field establishment. Similar results on the rooting and subsequent field establishment were also reported by Boxus (1974), Owen and Miller (1996), and Jimenez-Bermudez and Redondo-Nevado (2002). Then, the rooted plants were gradually acclimatized and transferred to the *ex vitro* condition for field evaluation (Fig. 1, H-J).

Table 2. Effect of rooting media formulation on root induction of *in vitro* regenerated shoots of strawberry. Data were recorded after 4 weeks of culture.

Treatment (mg/l)			Shoots induced root development (%)						
			AOG	JP-2	JP-3	Camarosa	Sweet Charly	Giant Mountain	Festival
Full strength	NAA	0.1	70	65	66	70	65	69	67
		0.5	90	86	85	88	85	85	88
		1.0	95	90	90	90	88	90	90
		2.0	91	90	90	90	90	90	90
	IBA	0.1	60	60	58	60	60	58	60
		0.5	88	87	87	88	80	80	80
		1.0	90	90	90	90	90	90	90
		2.0	88	85	84	83	83	85	83
Half strength	NAA	0.1	65	60	65	65	66	60	65
		0.5	65	60	60	60	63	63	60
		1.0	85	80	80	85	85	85	80
		2.0	55	55	56	57	56	55	56
	IBA	0.1	45	45	43	50	50	55	44
		0.5	60	57	58	58	60	57	58
		1.0	75	75	70	77	77	75	72
		2.0	60	60	59	60	58	60	60
Without growth regulators	½ MS0		96	95	95	96	95	92	92
	MS0		100	100	100	100	100	100	100

Field evaluation and data analysis

Among the seven strawberry varieties, 30-50 somaclones from each of the varieties were transplanted to the field and considered as Ro plants. In order to evaluate the somaclonal variation among the plants, data were recorded on different morphological and agronomical charactres. It was observed that regenerated plants grown in the field were not identical to their mother plant (Fig. 2, Tables 3-4). In the present study seven strawberry genotypes and their somaclones were evaluated for sixteen characters (eight morphological and eight yield contributing characters). Collected data were analyzed in order to estimate mean with standard error, analysis of variance (ANOVA), least significant difference (LSD) and coefficient of variability (CV%). In the analysis of variance the main item genotype was highly significant for all characters at 5% level of significance (Tables 3-4). These results indicate that genotypes were different from each other and justify their inclusion in the present investigation as materials. The replication items were non-significant for all characters. Haque (1997) obtained similar results in chickpea.

In the last part of this research, field evaluation of the seven strawberry varieties and their somaclones was conducted under different stress conditions: biotic (fungal diseases) and abiotic stress (temperature). Verticillium wilt, phytophthora crown rot, leaf scotch, leaf spot, leaf blight and botrytis fruit rot disease were found in the strawberry field (Fig. 3, A-F).

Table 3. Morphological characters of selected somaclones and comparison with their respective seven strawberry parents.

Varieties/ somaclones	Plant height (cm) (Mean±SE)	No. of leaves/plant (Mean±SE)	Petiole length (cm) (Mean±SE)	No. of stolons/ Plant (Mean±SE)	No. of nodes/ Stolon (Mean±SE)	Stolon length (cr (Mean±SE)	No. of crowns/ Plant (Mean±SE)	Canopy size (cm^2) (Mean±SE)
AOG	16.40±0.21	20.23± 0.14	14.23±0.14	10.07±0.43	4.27±0.14	120.4±0.35	3.20±0.058	364.23±8.14
AOG SC 1	21.47±0.58	26.40±0.21	16.07±0.26	14.23±0.14	4.90±0.21	161.5±13.8	6.50±0.057	474.40±0.26
AOG SC 2	28.00±0.29	25.27±0.15	15.40±0.20	12.43±0.23	4.40±0.15	155.0±0.40	5.40±0.057	374.37±0.30
AOG SC 3	31.97±0.32	30.33±0.08	17.23±0.14	16.37±0.18	6.27±0.14	163.1±0.20	7.50±0.057	594.03±0.52
AOG SC 4	17.40±0.21	20.47±0.14	15.47±0.14	13.27±0.14	5.27±0.14	145.0±0.11	5.50±0.057	473.87±0.63
LSD value (at 5% level)	2.2250	1.3252	1.8361	2.3382	1.3902	55.2871	0.5272	31.5607
CV%	1.8763	1.0495	2.2758	3.4236	5.3822	7.2115	1.8233	1.3446
JP-2	15.47±0.20	20.27±0.39	13.57±0.23	4.27±0.12	2.43±0.09	114.23±3.03	3.27±0.14	382.77±1.22
JP- 2 SC 1	24.00±0.29	22.67±0.12	15.23±0.14	5.33± 0.12	3.20±0.10	145.27±0.14	4.37±0.07	450.27±0.15
JP- 2 SC 2	22.23±0.21	24.50±0.17	15.33±0.17	5.83±0.067	3.30±0.11	155.33±0.24	6.47±0.09	474.40±0.14
LSD value (at 5% level)	0.5941	1.3090	1.3006	0.5286	0.2426	11.6129	0.7123	4.4029
CV%	0.7940	1.6006	2.4299	2.8243	2.2388	2.3083	4.1657	0.2777
JP-3	16.13±0.18	15.47±0.20	13.27±0.15	4.40±0.05	2.33±0.09	100.70±0.44	3.03±0.26	383.40±3.31
JP-3 SC 1	25.03±0.26	19.60±0.20	14.23±0.15	6.27±0.15	3.13±0.09	149.73±0.39	5.40±0.06	451.23±0.16
JP-3 SC 2	22.00±0.23	18.60±0.21	13.80±0.15	6.10±0.10	4.23±0.12	150.13±0.23	6.63±0.09	462.37±0.30
LSD value (at 5% level)	0.3536	1.3090	0.9394	0.7377	0.2101	1.8710	1.0147	11.6663
CV%	0.4616	2.0112	1.8755	3.6279	1.7856	0.3851	5.5531	0.7417
Camarosa	15.43±0.23	18.47±0.26	14.30±0.15	4.33±0.09	2.47±0.09	113.73±3.38	2.80±0.06	361.60±2.27
Camarosa SC 1	22.27 ±0.15	20.37±0.09	15.27±0.15	4.67±0.12	3.27±0.09	144.30±0.15	6.17±0.09	474.37±0.30
Camarosa SC 2	24.43 ±0.12	22.50±0.17	15.27±0.15	4.77±0.09	3.07±0.12	148.20±0.12	6.30±0.06	374.30±0.36
LSD value (at 5% level)	1.3174	1.3748	1.0573	0.6417	0.6643	12.3271	0.4113	8.8358
CV%	1.7483	1.8482	1.9445	3.8437	6.2241	2.5021	2.2213	0.6020
Sweet Charly	15.33 ±0.17	12.30±0.15	14.27 ±0.15	4.60 ±0.06	2.47±0.09	109.37 ±5.33	2.50 ±0.06	337.40±3.05
Sweet Charly SC 1	22.23±0.15	17.47±0.26	14.57±0.18	6.33±0.12	4.13±0.09	155.23±0.15	6.17±0.12	474.43±0.23
Sweet Charly SC 2	24.03±0.29	14.53±0.15	15.30±0.12	5.77±0.09	3.30±0.12	148.27±0.15	5.30±0.15	374.57±0.12
LSD value (at 5% level)	1.4779	0.6124	1.0362	0.5558	0.6967	18.8898	0.6752	11.6672
CV%	1.9783	1.1399	1.9360	2.7441	5.8026	3.7726	3.9865	0.8109
Giant Mountain	16.47±0.15	12.27±0.15	15.20±0.15	3.40 ±0.06	2.33 ±0.07	115.93 ±3.53	3.30 ±0.12	357.50 ±1.31
Giant Mountain SC 1	22.30±0.15	16.63 ±0.13	16.63±0.13	5.50±0.06	3.00±0.06	155.67±0.12	6.20±0.06	450.53±0.20
Giant Mountain SC 2	22.13±0.32	13.23±0.15	15.50±0.29	5.67±0.09	3.03±0.09	150.23±0.12	5.70±0.06	474.53±0.15
LSD value (at 5% level)	1.4247	1.0362	1.1279	0.4851	0.1918	12.8639	0.5753	4.5604
CV%	1.9294	2.0278	1.9649	2.7460	1.8898	2.5145	3.1207	0.2932
Festival	15.57±0.23	13.10 ±0.21	13.23 ±0.15	2.63 ±0.09	2.30 ±0.06	113.03 ±0.32	2.80 ±0.06	218.83±4.41
Festival SC 1	25.30±0.41	18.27±0.15	15.20±0.15	3.57±0.12	2.77±0.09	151.73±3.37	6.23±0.19	299.63±0.19
Festival SC 2	22.53 ±0.14	20.33±0.09	15.20±0.15	4.30±0.06	3.20±0.06	150.27±0.12	5.80±0.06	333.20±0.17
LSD value (at 5% level)	1.4169	1.0813	1.0573	0.6474	0.5073	12.4074	0.8489	16.0482
CV%	1.8428	1.7246	1.9980	5.0843	5.0604	2.4650	4.7191	1.5537

Table 4. Fruit yield and yield contributing characters of selected somaclones and comparison with their respective seven strawberry parents.

Varieties/ somaclones	Days to Flowering (Mean±SE)	No. of flower clusters/plant (Mean±SE)	No. of flowers/ Plant (Mean±SE)	No. of fruits/ Cluster (Mean±SE)	No. of fruits/plan (Mean±SE)	Days to fruit Harvest (Mean±SE)	Average fruit w (g) (Mean±SE)	Fruit wt./plant (Mean±SE)
AOG	67.67±0.33	6.33±0.33	15.67±0.33	2.67±0.33	9.67±0.33	90.33±0.33	25.30±0.15	244.33±9.67
AOG SC 1	61.67±0.33	7.67±0.33	17.67±0.33	4.00±0.33	15.33±0.33	84.67±0.33	30.03±0.14	460.43±8.28
AOG SC 2	62.00±0.33	6.33±0.33	17.67±0.33	4.33±0.57	17.67±0.57	84.00±0.33	28.23±0.14	498.80±10.04
AOG SC 3	60.00±0.33	8.67±0.33	21.67±0.33	6.33±0.33	20.33±0.35	81.67±0.33	38.00±0.11	772.60±10.67
AOG SC 4	61.67±0.33	7.33±0.33	18.67±0.33	3.67±0.33	15.00±0.58	82.67±0.33	30.13±0.23	442.73±7.99
LSD value (at 5% level)	2.3011	3.0440	3.0440	3.3871	3.7576	2.8954	1.0975	91.2854
CV%	0.7144	8.1414	3.2387	15.6733	4.6814	0.6646	0.7031	3.6672
JP-2	69.67±0.33	5.67±0.33	14.33±0.33	2.33±0.33	6.67±0.33	90.33±0.33	20.37±0.09	144.93±0.74
JP- 2 SC 1	61.33±0.33	7.67±0.33	17.67±0.33	4.33±0.33	14.33±0.33	84.67±0.33	32.17±0.73	504.33±2.89
JP- 2 SC 2	61.67±0.33	6.67±0.33	18.67±0.33	2.67±0.33	13.67±0.33	84.67±0.33	22.10±1.70	338.17±9.27
LSD value (at 5% level)	2.4255	2.1006	2.4255	2.4255	1.9176	2.4255	5.2738	31.1646
CV%	1.0381	8.6603	3.9474	21.4286	4.5610	0.7702	5.8266	2.6024
JP-3	70.00±0.33	6.00±0.33	14.33±0.33	2.00±0.33	6.67±0.33	92.67±0.33	22.27±0.15	154.57±0.43
JP-3 SC 1	61.67±0.33	7.67±0.33	17.67±0.33	2.67±0.33	14.33±0.33	83.67±1.20	31.17±0.65	482.27±3.05
JP-3 SC 2	61.67±0.33	6.67±0.33	18.67±0.33	6.00±0.57	14.33±0.33	84.67±0.33	32.43±0.23	497.03±2.57
LSD value (at 5% level)	1.9176	1.2128	2.4255	2.8442	1.2128	3.6383	1.7491	14.2024
CV%	0.8178	4.9180	3.9474	21.9863	2.8302	1.1494	1.6796	1.0328
Camarosa	70.00±0.33	5.33±0.33	16.33 ±0.33	2.00±0.33	5.33±0.33	90.33±0.33	21.90 ±1.55	132.67 ±5.36
Camarosa SC 1	61.67±0.33	5.67±0.33	18.33±0.33	3.00±0.33	13.67 ±0.33	84.33±0.67	31.03±0.20	438.37±1.44
Camarosa SC 2	61.00±0.58	6.67±0.33	18.67±0.33	6.67±0.33	13.33±0.33	85.00±0.33	32.43±0.09	426.60±2.01
LSD value (at 5% level)	1.9176	1.2128	2.4255	1.2128	2.4255	3.2087	5.7960	21.7791
CV%	0.8207	5.6604	3.7500	8.5714	6.1856	1.0189	5.5983	1.8001
Sweet Charly	71.67 ±1.66	5.33 ±0.33	15.33±0.33	2.00 ±0.33	6.00 ±0.33	90.33±0.33	23.10±0.23	144.00±1.53
Sweet Charly SC 1	61.67±0.33	6.33±0.33	18.33±0.33	2.67±0.33	14.33±0.33	84.67±0.33	31.93±0.87	461.33±2.62
Sweet Charly SC 2	61.67±0.33	7.00±0.58	17.33±0.33	3.00±0.33	13.67±0.33	84.33±0.33	23.37±0.44	334.50±0.58
LSD value (at 5% level)	7.2766	1.2128	2.1006	1.2128	1.4853	2.4255	4.0296	10.0375
CV%	3.0769	5.3571	3.3962	13.0435	3.6022	0.7712	4.2381	0.8806
Giant Mountain	70.33 ±0.33	5.67 ±0.33	15.33 ±0.33	2.00 ±0.33	4.67±0.33	90.33 ±0.33	23.37±0.19	122.67±3.71
Giant Mountain SC 1	62.00±0.33	6.67±0.33	18.33±0.33	2.67±0.33	13.33±0.33	84.33±0.33	32.23±0.12	433.80±0.31
Giant Mountain SC 2	61.67±0.33	6.33±0.33	17.00±0.58	3.00±0.33	13.67±0.33	84.67±0.33	32.60±0.15	463.43 ±1.62
LSD value (at 5% level)	1.4853	2.4255	3.2087	1.2128	2.4255	2.4255	0.5355	14.4131
CV%	0.6313	10.7143	5.2219	13.0435	6.3158	0.7712	0.5007	1.1653
Festival	75.00±0.58	5.33±0.33	13.00 ±0.58	2.00 ±0.33	5.67±0.33	90.33 ±0.33	21.67 ±0.07	138.87±5.88
Festival SC 1	62.00±0.58	6.67±0.33	16.00±0.58	2.33±0.33	5.67±0.33	84.67±0.33	30.93±0.59	445.83±1.09
Festival SC 2	61.67±0.33	7.00±0.58	15.67±0.33	3.00±0.33	12.67±0.33	84.67±0.33	31.83±0.87	429.23±0.80
LSD value (at 5% level)	3.8351	2.9707	3.2087	1.2128	2.1006	2.4255	4.6751	22.4657
CV%	1.5918	12.8921	5.9233	13.6364	5.4127	0.7702	4.5656	1.8270

Fig. 2. Different plant types (A-H) and fruit shapes (I-P) showing somaclonal variations.

Among the six strawberry diseases, disease incidences (%) of verticillium wilt and phytophthora crown rot was high (60%) in seven donor parent's viz. AOG, JP-2, JP-3, Camarosa, Sweet Charly, Giant Mountain and Festival but in somaclones it was 15% (Table 5). Other diseases *viz*. leaf scotch, leaf spot, leaf blight and botrytis fruit rot, disease incidence (%) was high (45-50%) in donor plants but in their somaclones it was low (10%). Most of the plants were severally affected with these diseases during the summer and were perished. There were no plants found resistance to fungal diseases.

High temperature one of the major factor that affects strawberry cultivation. During the summer month April-May temperature becomes high (above 38°C) and the plants do not perpetuate in the field. In terms of summer overcoming capacity, majority of plants were found heat sensitive in donor plants. In their somaclones, 75-80% plants showed moderate summer overcoming capacity and 15-20% plants showed high summer overcomming capacity. Somaclone AOG SC 3 showed better performance than other somaclones and donor parents in terms of summer overcoming capacity (Table 6). These somaclones can be acceptable commercially if the good characters exhibited are transmitted through generations or could be used in future breeding programme for the improvement of strawberry varieties in Bangladesh.

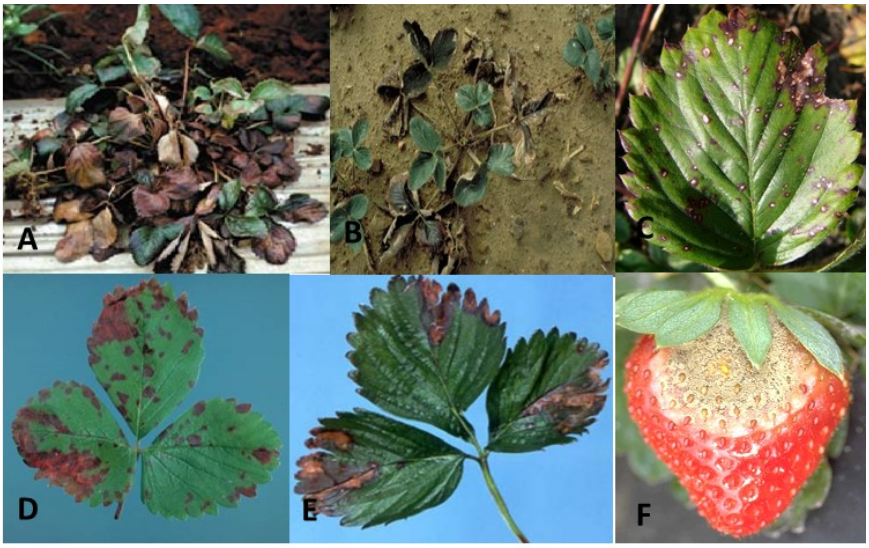

Fig. 3. Phytophthora crown rot disease of strawberry (A), strawberry plant dying from Verticillium wilt (B), strawberry leaf spot symptoms on leaflet (C), leaf scotch on strawberry (D), Pomopsis leaf blight on strawberry (E) and Botrytis fruit rot on mature strawberry fruit (F).

Table 5. Disease incidence (%) of seven strawberry cultivars and their somaclones. Data were recorded after 90-120 days after plantation in the field.

Cultivers/ Somaclones	Disease incidence (%)					
	Verticillium wilt	Phytophthora crown rot	Leaf scotch	Leaf spot	Leaf blight	Botrytis fruit rot
AOG	60	60	50	50	50	45
AOG SC 1	15	10	10	10	10	10
AOG SC 2	15	15	10	10	10	10
AOG SC 3	10	10	00	00	00	00
AOG SC 4	15	15	10	10	10	10
JP-2	60	60	50	50	50	50
JP-2 SC 1	15	15	10	10	10	10
JP-2 SC 2	15	15	10	10	10	10
JP-3	60	60	50	50	50	50
JP-3 SC 1	15	15	10	10	10	10
JP-3 SC 2	15	15	10	10	10	10

Table 5 Contd.

Camarosa	60	60	50	50	50	50
Camarosa SC 1	15	15	10	10	10	10
Camarosa SC 2	15	15	10	10	10	10
Sweet Charly	60	60	50	50	50	50
Sweet Charly SC 1	15	15	10	10	10	10
Sweet Charly SC 2	15	15	10	10	10	10
Giant Mountain	60	60	50	50	50	50
Giant Mountain SC 1	15	15	10	10	10	10
Giant Mountain SC 2	15	15	10	10	10	10
Festival	60	60	50	50	50	50
Festival SC 1	15	15	10	10	10	10
Festival SC 2	15	15	10	10	10	10

Table 6. Summer overcoming potentiality of seven strawberry varieties and their somaclones. Data were recorded 120 days after plantation in the field.

Varieties/Somaclones	Summer overcoming potentiality (%)		
	Low (28-30°C)	Moderate (30-35°C)	High (above 35°C)
AOG	95	5	--
AOG SC 1	10	75	15
AOG SC 2	10	75	15
AOG SC 3	--	80	20
AOG SC 4	10	75	15
JP-2	95	5	--
JP-2 SC 1	10	75	15
JP-2 SC 2	10	75	15
JP-3	95	5	--
JP-3 SC 1	10	75	15
JP-3 SC 2	10	75	15
Camarosa	95	5	--
Camarosa SC 1	10	75	15
Camarosa SC 2	10	75	15
Sweet Charly	95	5	--
Sweet Charly SC 1	10	75	15
Sweet Charly SC 2	10	75	15
Giant Mountain	95	5	--
Giant Mountain SC 1	10	75	15
Giant Mountain SC 2	10	75	15
Festival	95	5	--
Festival SC 1	10	75	15
Festival SC 2	10	75	15

Acknowledgement

The authors thankfully acknowledge the Ministry of Science and Technology (MOST) of the Peoples Republic of Bangladesh for financial support for this study.

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J. Bio-Sci. 24: 33-43, 2016 ISSN 1023-8654
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RAPID DETERIORATION OF WETLAND IN BANGLADESH: A SCENARIO OF NOIKANDI *BEEL*

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Abstract

Wetland aggregates at least a part of human heritage. It has direct impact on human culture and society. Besides, wetland comprises significant components in the context of local and national biodiversity. *Beel* wetland is a unique environment of floral and faunal association. In Bangladesh, wetland plays a vital role in its economic, industrial, ecological, socioeconomic and cultural attitudes. A study was conducted in the Noikandi *beel* where rapid destruction was observed due to anthropogenic activities. In the present study, seasonality and macro aquatic diversity and of the Noikandi *beel* Bangladesh was examined. Plant materials were collected from the *beel* once in a month for a period of 24 months. In the first 12 months, a total of 52 taxa have been recorded. Among them 40.38% was aquatic, 46.77% amphibians and 13.46% were found to be terrestrial. In addition, a rare taxon *Euryale ferox* Salisb was found. In the consecutive year of the study, 22.03% were aquatic, 52.54% terrestrial, 22.03% amphibians and1.69% was found to be macro algae. The rapid disappearance of aquatic plants is alarming. On the contrary, a good number of plants have been noted that is not associated with wetland environment indicating the transformation of wetland in to terrestrial ecosystem. Along with declining water depth and siltation other anthropogenic activities, agricultural, developmental initiatives have been identified as major threats, towards the Noikandi *beel*. The recommendations of the present study could be implemented by the government with the help of local people, as well as, this information may be useful for other *beels* of Bangladesh.

Key words: Bangladesh, diversity, Noikandi *beel*, policy, rapid deterioration, wetland

Introduction

Bangladesh is a well-known country overlapped by numerous *beels*, *haors*, *baors*, rivers, lakes, tributaries, floodplains and ponds (Islam and Wahab 2005, Alam et al. 2014). As usual, the wetlands are famous for its biodiversity (Nishat et al. 1993, Davis and Froend 1999, Islam and Wahab 2005). Almost 50% of the total land surface are wetland including *beels*, *haors*, *baors*, rivers, estuaries, mangrove swamps, and water storage reservoirs, fish ponds and some other lands that are seasonally flooded to a depth of about 30 cm or more than 30 cm (Islam and Gnauck 2008).

The village people exclusively rely on the natural resources for their food, shelter, fuel, medicine or any other means of daily subsistence in Bangladesh. Almost 50% of the people of Bangladesh are directly reliant on wetlands resources. The majority of the deprived people in the wetlands areas are directly or indirectly depend on the resources for their nutrition (Davis and Froend 1999)

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Wetlands have great impact and have been well established as an active force for floral and faunal conservation as well as rural socioeconomic improvement. The widespread loss and degradation of wetlands is remarkable in the recent decade (Russi et al. 2013). Therefore, degradation or sustainable existence of the resources impacts on livelihood security to a great extent (Baland and Platteau 1996, Scherr 2000). In addition, the wetland comprises very rich components of biodiversity in the context of local, national, and regional implication. They also make available habitat for a diversity of resident and migratory waterfowl, a significant number of endangered species, as well as a large number of commercially important species (Islam 2010, Webb et al. 2010).

More than 350 species have been noted as weeds of the cultured field including wetlands. The number of species in an area be subject to the land usage patterns and its ecological circumstances (Gaston 2000). In *beels*, *haors*, *baors*, ditches where no crop cultivation is experienced, a large number of aquatic plants grow and form thick natural vegetation. Recently, awareness regarding wetland as a resource of living security is a concern to the populace. Diverse human actions are nowadays a key force upsetting the all ecosystems worldwide (Vitousek et al. 1997, Sala et al. 2000).

The present study was aimed to evaluate the seasonality, diversity and status of the plants of the Noikandi *beel*, Natore, Bangladesh. Moreover, the causes of wetland degradation and destruction along with their possible conservation strategy have been focused.

Materials and Methods

The study was carried out from April 2014 to April 2016 to collect all the plant materials from the Noikandi *beel* located at Natore Sadar upazilla (88° 56' 24.41" N, 88.93°E) beside the Natore Paurosava, Natore, Bangladesh. It is approximately 2 kilometers long and 1 kilometer width. The *beel* is connected with the river Barnoi, which is the outlet river of the Atrai. Major parts of the *beel* are plain land but the middle position of the *beel* is deep, where the water retains round the year. The inundation depth during the flood period differs from 1-5 meters. Total 500m areas have been thoroughly searched (Fig. 1 A, B). Specimens were sampled by hand from the *beel* from 5 to 20 cm depth of water. An anchor tied with rope was used to assemble the specimen from greater depth. Plants were agitated underwater to remove the bulk of loose detritus and stored for transportation in the plastic vial containing the *beel* water.

In the laboratory, specimens were washed with double distilled water, preserved in Transeau's solution (Transeau 1916) and also preserved as dry herbarium specimens. The herbarium sheets have been conserved at the Phycology and Limnology Laboratory, Department of Botany, University of Rajshahi, Bangladesh. Fresh specimens were cautiously teased out and arranged in distilled water on a white tray.

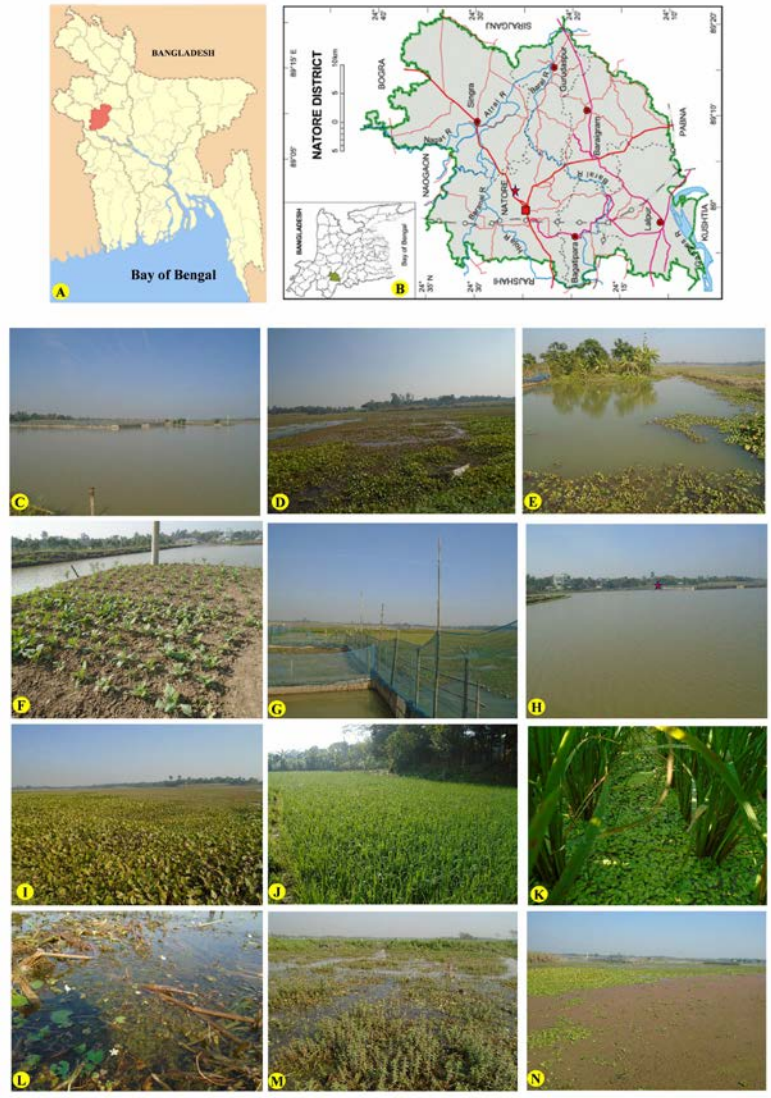

Fig. 1. (A) Map of Bangladesh indicating Natore district, (B) Map presents Natore city in the Natore district, Bangladesh, (C) Parts of Noikandi *beel* wetland with the artificial water supply, (D) Hydrophytes with other vegetation, (E) Parts of the beel where a current jal (fine net) is drying for the fishing, (F) Encroachment and Tomato cultivation inside the *Beel*, (G) Artificial barrier for fish culture adjacent with the *beel* water, (H) The red point indicate an animal farm a biscuit factory near the *beel*, (I) Massive *Echinochloa* spp. are disturbing the regular flora, (J) Rice cultivation alongside the beel, (K) Algae and other amphibians in the rice field inside the *beel*, (L) Charophyceae and Nymphaeaeceae were observed during the rainy season, (M) *Enhydra* sp. with other vegetations, (N) Parts of the *beel* with algal bloom and hydrophytes indicating eutrophication.

Results and Discussion

During the first year of the present investigation (April 2014 - March 2015) a total of 52 taxa were noted (Table 1); among these 40.38% were aquatic, 46.15% amphibian and 13.46% were found to be terrestrial. It appears that terrestrial plants are occupying wetland habitat that represents the threatened status of the wetland (Table 1). Seasonality of the collected plants was noted. Some of them were present throughout the year and others occurred only in particular season (Table 1). Throughout the period of study, the regular depth of water of the *Beel* was not more than 150 cm.

In case of diversity, the members of cyperaceae dominated over the rest followed by characeae and euphorbiaceae (Table 1). Twelve families had only sole representative out of 21 families. During the study period *Euryale ferox* Salisb. was only found in 2014. *E. ferox* is credited to absorb the toxic heavy metals. As a consequence, it could be a reason for the less richness and for the short seasonality of this plants (Rai et al. 2002). *Nelumbo* spp. only originated during rainy and autumn season. This plant exhibited sporadic abundance indicating its declining population in this wetland. Eutrophication, as well as acidification of water bodies, may eventually consequence in the total disappearance of all aquatic macrophytes with the exception of the floating-leaved Nymphaeids *Nymphaea* spp. (Arts et al. 1990). Likewise, we observed the rich abundance of *Nymphaea* sp. among the members of Nymphaeaceae throughout the study period.

In the first year of study, charophytes were not found during summer, autumn, late autumn, winter and spring. During these seasons, almost the whole *beel* area goes under agricultural practices. In the rainy season, the deepest zone of the wetland is kept free from agricultural practices. Only in the post monsoon i.e. during mid September to late October charophytes were found with other aquatic vegetation (Table 1) in the deepest zone of the wetland concerned. In general, charophytes are found in Bangladesh all the year round (Naz et al. 2009, Naz et al. 2011, Diba et al. 2013) but during the present study, charophytes were recorded during rainy season indicating their threatened existence in this wetland due to agricultural practices. On the contrary, a charophyte was recorded for the first time from Bangladesh (*Nitella axillaris* Braun) during this study. In a similar study performed to assess the biodiversity status of a wetland ecosystem, Mohangonj Upazila in Netrakona district of Bangladesh concluded with the findings that the wetland diversity is at a life-threatening risk due to major environmental threats (Alam 2014). As evident, the water depth of this wetland is drastically getting lesser during longer dry seasons and in post monsoon too; causing the nearly threatened existence of deep rooted hydrophytes in this wetland.

In the second year of the study, the 52.54% were terrestrial, 22.03% were amphibious, 1.69% was macro algae and the rest were other macrophytes of different groups (Table 2). Most of the wetland plants have not been noted with the higher abundance that was found in the first year of study. On the other hand, some new plants have been recorded, indicating the present status of the *beel* (Table 2). Numbers of taxa belonging to poaceae family have been increased. This indicate that the beel is being transforming to terrestrial environment (Rosa et al. 2009).

According to Gallagher et al. (2003) dominance of Asteraceae realm the terrestrial environment. During present study 8 taxa of Asteraceae were observed indicating the transformation wetland towards terrestrial ecosystem; In addition, during the second-year study, we found only one species of charophyceae whereas in the first year we recorded 8 taxa. This information indicates that the biodiversity of this beel is rapidly declining due to enhanced eutrophication; because it is commonly known that charophyte can flourish well in clean water (Naz et al. 2011).

Table 1. Checklist and seasonal distribution of vegetation of the Noikandi *beel* (2014-2015).

Family	Taxon	Habit	Habitat	Seasonal distribution					
				Summer 2014	Rainy 2014	Autumn 2014	Late-autumn L.Autun 2014	Winter 2015	Spring 2015
	Oryza sativa L.	Monocot	Aquatic	+	+	+	+	+	+
	Cynodon dactylon L.	Monocot	Terrestrial	+	_	+	+	+	+
Poaceae	*Echinochloa colonum* L.	Monocot	Amphibian	+	_	+	+	+	+
Cyperaceae	*Schoenoplectus grossus* L.	Monocot	Amphibian	+	+	+	+	+	+
	Cyperus difformis L.	Monocot	Amphibian	+	+	+	+	+	+
	Cyperus michelianus L.	Monocot	Amphibian	+	+	+	+	+	+
	Cyperus tuberosus Rottb.	Monocot	Amphibian	+	+	+	+	+	+
	Cyperus rotundus L.	Monocot	Amphibian	+	+	+	_	_	_
	Eleocharis acutangula Roxb.	Monocot	Amphibian	_	+	+	_	_	_
	Fimbristylis schoenoides Retz.	Monocot	Amphibian	_	+	+	_	_	_
	Fuirena ciliaris (L.) Roxb.	Monocot	Amphibian	_	+	+	_	_	_
	Kyllinga brevifolia Roxb.	Monocot	Amphibian	_	+	+	_	_	_
	Schoenoplectus articulates (L.) Palla	Monocot	Amphibian	_	+	+	_	_	_
	Schoenoplectus juncoides (Roxb.) Palla	Monocot	Amphibian	_	+	+	_	_	_
Eriocaulaceae	*Eriocaulon setaceum* L.	Monocot	Amphibian	_	+	+	_	_	_
Hydrocharitaceae	*Hydrilla verticillata* (L.F.) Royle	Monocot	Aquatic	_	+	+	_	_	_
	Ottelia alismoides (L.) Pers.	Monocot	Amphibian	_	+	+	_	_	_
	Vallisneria spiralis L.	Monocot	Amphibian	_	+	+	_	_	_
Lemnaceae	*Lemna* L.	Monocot	Aquatic	_	+	+	_	_	_
	Spirodela polyrhiza L.	Monocot	Aquatic	_	+	+	_	_	_
Najadaceae	*Najas graminea* Del.	Monocot	Aquatic	_	+	+	_	_	_
	Potamogeton mucronatus Presl.	Monocot	Aquatic	_	+	+	_	_	_
Pontederiaceae	*Eichhornia crassipes* Mart.	Monocot	Amphibian	_	+	+	_	_	_
	Monochoria hastata L.	Monocot	Amphibian	_	+	_	_	_	_
Alismataceae	*Sagittaria guayanensis* Kunth	Monocot	Amphibian	_	+	_	_	_	_
Salviniaceae	*Salvinia natans* (Linn.) All.	Pteridophyta	Amphibian	_	+	_	_	_	_

Table 1 Contd.

Azollaceae	*Azolla pinnata* R.Br.	Pteridophyta	Amphibian	+	+	+	+	+	+
Marsileaceaceae	*Marsilea minuta* L.	Pteridophyta	Amphibian	_	+	_	_	_	_
Parkeriaceae	*Ceratopteris pteridoides* (Hook.) Hiern.	Pteridophyta	Amphibian	_	+	_	_	_	_
Ceratophyllaceae	*Ceratophyllum demersum* L.	Dicot	Terrestrial	+	+	+	+	+	+
Asteraceae	*Enhydra fluctuans* Lour.	Dicot	Amphibian	+	+	+	+	+	+
Convolvulaceae	*Ipomoea aquatica* Forsk.	Dicot	Aquatic	_	+	_	_	_	_
Lentibulariaceae	*Utricularia inflexa* Forsk.	Dicot	Aquatic	_	+	_	_	_	_
Menyanthaceae	*Nymphoides aurantiacum* Dalz.	Dicot	Aquatic	_	+	_	_	_	_
Nymphaeaceae	*Nymphaea nouchali* Burm.f.	Dicot	Aquatic	_	+	_	_	_	_
	Nelumbo nucifera Gaertn.	Dicot	Aquatic	_	+	+	_	_	_
	Euryale ferox Salisb.	Dicot	Aquatic	_	+	+	_	_	_
Onagraceae	*Ludwigia adscendens* (L.) Hara.	Dicot	Amphibian	+	_	_	+	+	+
Euphorbiaceae	*Euphorbia hirta* L.	Dicot	Terrestrial	+	_	_	+	+	+
	Chenopodium album L.	Dicot	Terrestrial	+	_	_	+	+	+
	Amaranthus spinosus L.	Dicot	Terrestrial	+	_	_	+	+	+
	Amaranthus viridis L.	Dicot	Terrestrial	+	_	_	+	+	+
	Vicia sativa L.	Dicot	Terrestrial	+	_	_	+	+	+
	Heliotropium indicum	Dicot	Terrestrial	_	+	_	_	_	_
Charophyceae	*Chara corallina* Klein ex Willd.	Algae	Aquatic	_	+	_	_	_	_
	C. setosa Klein ex Willd.	Algae	Aquatic	_	+	_	_	_	_
	C. zeylanica var. *sejuncta* (A,Br.) R.D.W.	Algae	Aquatic	_	+	_	_	_	_
	Nitella hyalina (DC.) Agardh	Algae	Aquatic	_	+	_	_	_	_
	Nitella axillaris Braun	Algae	Aquatic	_	+	_	_	_	_
	Lychnothamnus barbatus (Meyen) Leonhardi	Algae	Aquatic	_	+	_	_	_	_
	N. furcata var. *mucronata*	Algae	Aquatic	_	+		_	_	_
	Chara fibrosa Ag. ex Bruuz	Algae	Aquatic	_	+	_	_	_	_

Table 2. Checklist and seasonal distribution of vegetation of the Noikandi *beel* (2015-2016).

Family	Taxon	Habit	Habitat	Seasonal distribution					
				Summer 2015	Rainy 2015	Autumn 2015	Late-Autumn 2015	Winter 2016	Spring 2016
Poaceae	*Oryza sativa* L.	Monocot	Aquatic	+	+	+	+	_	_
	Cynodon dactylon L.	Monocot	Terrestrial	+	+	+	+	+	+
	Echinochloa colonum L.	Monocot	Terrestrial	+	+	+	+	+	+
	Pennisetum purpureum Schum.	Monocot	Terrestrial	+	+	+	+	+	+
	Saccharum officinarum L.	Monocot	Terrestrial	+	+	+	+	+	+
	Saccharum spontaneum L.	Monocot	Terrestrial	_	+	+	+	_	_
	Dactyloctenium aegyptium (L.) P. Beauv.	Monocot	Terrestrial	+	+	+	+	+	+
	Hygroryza aristata (Retz.) Nees	Monocot	Terrestrial	_	+	+	+	+	_
Pontederiaceae	*Eichhornia crassipes* (Mart.) Solms	Monocot	Aquatic	_	+	+	+	+	_
	Monochoria hastata (L.) Solms	Monocot	Aquatic	_	+	+	+	+	_
Potamogetonaceae	*Potamogeton natans* L.	Monocot	Aquatic	_	+	+	+	+	_
Cyperaceae	*Cyperus difformis* L.	Monocot	Amphibian	+	_	_	_	+	+
	Cyperus michelianus L.	Monocot	Amphibian	+	_	_	_	+	+
	Cyperus tuberosus Rottb.	Monocot	Amphibian	+	_	_	_	+	+
	Cyperus rotundus L.	Monocot	Amphibian	+	_	_	_	+	+
	Eleocharis acutangula Roxb.	Monocot	Amphibian	_	+	+	_	_	_
	Fimbristylis schoenoides Retz.	Monocot	Amphibian	+	_	_	+	+	+
	Schoenoplectus articulates (L.) Palla	Monocot	Amphibian	_	+	+	+	+	_
	Schoenoplectus juncoides (Roxb.) Palla	Monocot	Amphibian	_	+	+	+	+	_
Hydrocharitaceae	*Ottelia alismoides* (L.) Pers.	Monocot	Aquatic	_	+	+	+	+	_
	Vallisneria spiralis L.	Monocot	Aquatic	_	_	_	_	_	_
Lemnaceae	*Lemna perpusilla* Torrey	Monocot	Aquatic	_	+	+	_	_	_
	Spirodela polyrhiza L.	Monocot	Aquatic	_	+	+	_	_	_
Salviniaceae	*Salvinia natans* (Linn.) All.	Pteridophyta	Aquatic	_	+	+	+	+	_
Azollaceae	*Azolla pinnata* R.Br.	Pteridophyta	Aquatic	_	+	+	+	+	_
Boraginaceae	*Heliotropium indicum*	Dicot	Terrestrial	+	_	_	_	+	+
Marsileaceae	*Marsilea minuta* L.	Pteridophyta	Amphibian	+	+	+	+	+	+
Ceratophyllaceae	*Ceratophyllum demersum* L.	Pteridophyta	Amphibian	+	+	+	+	+	+
Asteraceae	*Enhydra fluctuans* Lour.	Dicot	Amphibian	+	+	+	+	+	+
	Ageratum conyzoides L.	Dicot	Terrestrial	+	_	_	_	+	+
	Cirsium arvense (L.) Scop	Dicot	Terrestrial	+	_	_	_	+	+
	Eclipta alba (L.) Hassk	Dicot	Terrestrial	+	_	_	_	+	+
	Mikania cordata (Burm.f.) Robinson	Dicot	Terrestrial	+	_	_	_	+	+
	Tridax procumbens L.	Dicot	Terrestrial	+	_	_	_	+	+
	Vernonia petula (Dryand.) Merr.	Dicot	Terrestrial	+	_	_	_	+	+
	Xanthium indicum Koen. ex Roxb.	Dicot	Terrestrial	+	_	_	_	+	+

Table 2 Contd.

Acanthaceae	*Acanthus auriculata* Schumach	Dicot	Terrestrial	+	+	+	+	+	+
Convolvulaceae	*Ipomoea aquatica* Forsk.	Dicot	Amphibian	+	+	+	+	+	+
Menyanthaceae	*Nymphoides aurantiacum* Dalz.	Dicot	Aquatic	_	+	+	+	+	_
Moraceae	*Ficus hispida* L.f.	Dicot	Terrestrial	+	+	+	+	+	+
Musaceae	*Musa sapientum* L.	Dicot	Terrestrial	+	+	+	+	+	+
Nymphaeaceae	*Nymphaea nouchali* Burm.	Dicot	Aquatic	_	+	+	+	+	_
Onagraceae	*Ludwigia adscendens* (L.) Hara.	Dicot	Amphibian	_	+	+	+	+	_
Euphorbiaceae	*Euphorbia hirta* L.	Dicot	Terrestrial	+	_	_	_	+	+
Fabaceae	*Vicia sativa* L.	Dicot	Terrestrial	+	_	_	_	+	+
	Lablab purpureus (L.) Sweet	Dicot	Terrestrial	+	_	_	_	+	+
Chenopodiaceae	*Chenopodium alba* L.	Dicot	Terrestrial	+	_	_	_	+	+
Amaranthaceae	*Amaranthus viridis* L.	Dicot	Terrestrial	+	_	_	_	+	+
	Amaranthus spinosus L.	Dicot	Terrestrial	+	_	_	_	+	+
Anacardiaceae	*Mangifera indica* L.	Dicot	Terrestrial	+	+	+	+	+	+
Rutaceae	*Citrus aurantifolia* (Christm. and Panzer) Swingle	Dicot	Terrestrial	+	+	+	+	+	+
Scrophulariaceae	*Limnophila heterophylla* (Roxb.) Benth., Scroph	Dicot	Amphibian	_	+	+	+	_	_
	Limnophila indica (L.) Druce	Dicot	Terrestrial	_	+	+	+	_	_
Verbenaceae	*Lippia alba* (Mill.) Briton et Wilson	Dicot	Terrestrial	+	+	+	+	+	+
	Phyla nodiflora (L.) Greene	Dicot	Terrestrial	+	_	_	_	+	+
Solanaceae	*Physalis minima* L.	Dicot	Terrestrial	+	_	_	_	+	+
	Physalis angulata L.	Dicot	Terrestrial	+	_	_	_	+	+
	Capsicum frutescens L.	Dicot	Terrestrial	+	_	_	_	+	+
Charophyceae	*Nitella furcata* var. *mucronata*	Algae	Aquatic	_	+	+	+	+	_

The information gap and threats

Inhabitants of large vertebrates may not be precise indicators of the status of all of freshwater species, but there are grounds for grave concern if their rank were reflected in even 5% of the total species complement. To date, however, there has been no comprehensive global analysis of freshwater biodiversity equivalent to those recently accomplished for terrestrial systems. Existing data on the population status or destruction rates of freshwater biota are prejudiced in terms of geography, territory types and taxonomy; most populations and habitats in some regions have not been supervised at all. Even a elementary global mapping of inland waters, classified by broad geomorphic categories, is deficient and therefore, there are no global estimates of changes in the extent of lakes, rivers or wetlands (Myers et al. 2000, Balmford et al. 2002, Dudgeon et al. 2006).

Management efforts for freshwater biodiversity are controlled by the fact that majority of the species in diverse communities are sporadic and thus their natural histories tend to be elusive. This indicates that overall species numbers are predictable; predicting the identities of the affected taxa is not possible.

In this study, the major threat was noted by the author's field visit and personal observation for the recent past few years from the study area. According to opinion and remarks of the local people, relevant literature,

and the present investigation, aquatic diversity was being drastically reduced due because of the massive agricultural practices (Fig. 1). As a normal procedure of agricultural practice, the farmers are using herbicides and pesticides to protect their crops.

Regular fertilization and irrigation is a common phenomenon for the agricultural practice. Both of the aforesaid practice in the wetland is also hampering the lifecycle of the aquatic plant diversity. Of note, the wetland area goes under drought for few months that directly affect the biodiversity of the wetland. Since there is less water in the wetland, the local people do the over fishing, in particular, during late autumn and early winter as well as thriving in the mud for collecting the fishes. In the *beel* area, there has been residential/commercial development that influence directly or indirectly to this *beel*.

Alien invasive species is another concern for the regular flora and fauna. In this *beel* presence of terrestrial alien invasive species *Cyperus rotundus* L. and *Ageratum conyzoides* L. were recorded during last year of investigation. Furthermore, indiscriminate utilization, encroachments for fishing or agricultural cultivation (Fig. 1) and urbanization have created negative impacts on the *beel*. The household effluent regularly discharged to the *beel*. For the artificial fish culture in the *beel*, people use rotten rice, different types of dung, rotten oilcake that may not be good for the other species which does also increases the accelerate the rate of eutrophication. Apart this, a biscuit factory and an animal farm have recently been established near the *beel* which produces organic wastes which are released in the beel water resulting change in the trophic status of the waterbody and shrinking the floral as well as faunal diversity of the concerned.

Recommendations for management of the Noikandi *beel*

Suitable use of wetlands can resolve the ecosystems problems in the wetland areas. The country needs an adequate scientific guideline, appropriate interdisciplinary policy and political commitment to implement it, for sustainable management and protection of wetlands e.g. *beels* and ecologically sensitive wetland ecosystems in Bangladesh. Thus, a consistent data bank is important what is provided in this study to improve the conservation measures initiated by the Government.

In the Fig. 1G, it was evident that artificial fish cultivation was going on inside the *beel* that has created a continuous negative impact on this *beel*. This artificial barrier should be removed as soon as possible. For the fish cultivation, farmers use deep tube well throughout the year that disturbs the natural growth and occurrence of the flora. Therefore, if there is a need for water to supply that should be by a scientific way.

Increasing awareness, understanding about the *beel* ecosystem, to the fishermen as well as to local people are the fundamental steps that may be employed through educational and cultural institution in the locality. The use of current jal (fine mesh net) should completely be prohibited. There are some scientific ways to manage the hydrophytes that can be applied manually. Around the *beel* area, there is a huge of agricultural cultivation. In case of fertilization, indiscriminate use of fertilizers should be stopped and the use of inorganic fertilizer should gradually be replaced by organic manure. After observing the siltation stage, there can be dug after particular time interval to prevent siltation. Agricultural practices must be practiced in a controlled way that does not harm the existence of the wetland. Use of pesticides and herbicides should be controlled and minimized as wetlands are one of the intricate, delicate and fragile ecosystems of Bangladesh its values yet to be explored by all. Any sort of drastic initiatives in or nearby wetlands should be safeguarded. Integrating the indigenous knowledge with the scientific knowledge could be helpful for the sustainable management of the wetland concerned.

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J. Bio-Sci. 24: 45-53, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

MULTIVARIATE STATISTICAL TECHNIQUES FOR METAGENOMIC ANALYSIS OF MICROBIAL COMMUNITY RECOVERED FROM ENVIRONMENTAL SAMPLES

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Abstract

High-throughput big dataset generated through next generation sequencing (NGS) of DNA samples helps identify key differences in the function and taxonomy between microbial communities as well as shed light on the diversity of microbes, cooperation and evolution in any particular ecosystem. During this study, three statistical techniques namely, Random Forest (RF), Multidimensional Scaling (MDS) and Linear Discriminant Analysis (LDA) approaches were employed for functional analysis of 212 publicly available metagenomic datasets within and between 10 environments against 27 metabolic functions. RF generates the 8 most important metabolic variables along with MDS and LDA among which Photosynthesis has the highest score (70.20); Phages, prophages has the second highest score (61.31) and Membrane Transport was found to have the eighth highest score (45.29). The MDS plot was found useful to visualize the separation of the microbes from human or animal hosts from other samples along the first dimension and the separation of the aquatic and mat communities along the second dimension. LDA analyses compared the extent of the microbial samples into three broad groups: the human and animal associated samples, the microbial mats, and the aquatic samples. RF showed that phage activity is a major difference between host-associated microbial communities and free-living. The MDS and LDA techniques suggest that mat communities were unique from both the animal associated metagenomes and the aquatic samples with differences in the vitamin and cofactor metabolism.

Key words: Environment, functional role, linear discriminant analysis, metagenomes, multiple dimensional scaling, random forest

Introduction

Most life on this planet is microbes that help to maintain the ecological balance greatly through their direct and indirect interactions with biotic and abiotic components of the environment. Metagenomics has recently started contributing to reveal the actual scenario surrounding biodiversity of this microbial life. The technique comprises extracting and sequencing the DNA and RNA (metatranscriptomics) of microbial communities collected directly from any specific samples e.g., human or plant/animal-associated, environmental, industrial, food sources and then using high performance computational and statistical analysis to associate function to each sequence (Dinsdale et al. 2013). Due to rapid advancement in IT as well as the continued and dynamic development of faster next generation sequencing technologies with various platforms, it is now a powerful tool to sequence multiple samples with millions of short DNA fragments or reads in a single run. This ultimately facilitates studying the multiple microorganisms living in an environmental community without the need of isolating and culturing individual microbial species in a laboratory. It has been reported that more than 99% out of the millions of microbial species known to exist on earth cannot be cultured in a laboratory

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(Huson et al. 2009). However one can attempt to generate new NGS metagenomic dataset with a view to detect organisms of similar nature. Further annotation of a possible metagenome is conducted by comparing the sample DNA to the sequences that are available in various databases such as NCBI, SEED, MG-RAST, or COG (Aziz et al. 2008, Wooley et al. 2010). In most cases, the DNA sequences similar to each corresponding protein are identified; therefore a metagenome provides information on the taxonomic makeup and metabolic potential of a microbial community (Tringe et al. 2005). However, one should be aware that if new sequences are found, they might not give any hit which needs further analyses to claim novel and previously unknown organisms.

Until now, most of the focus in metagenomics has been on single environments such as coral atolls (Wogley et al. 2007, Dinsdale et al. 2008), cow intestine (Brulc et al. 2009), ocean water (Angly et al. 2006), and microbialites (Breitbart et al. 2009). Early work compared extremely different environments like soil microbes compared to water microbes (Breitbart et al. 2009). More recently, the Human microbiome project (https://hmpdacc.org) has expanded our understanding of the microbes inhabiting our own bodies, comparing samples from the same site among and between individuals (Tunrbaugh et al. 2010). These studies reflect the dynamic and expanding field of metagenomics which has been shown elsewhere (Wogley et al. 2007). Metagenomics provides a complete analysis of the microbial activity in terms of how the microbial community or metabolic potentials (of a group of organism) vary between sampling locations or at different time points (Kurokawa et al. 2008). During this study attempt has been made to explore the abilities of metagenomics technique while analyzing the metabolic profile of microbial communities with eventual visualization of large amounts of multivariate data.

Materials and Methods

A total of 212 metagenome datasets were selected from publicly available database (https://dinsdalelab.sdsu.edu/metag.stats/).These were classified into 10 different environments depending on the descriptions provided by the researcher who submitted the data. The experiments were involved a number of NGS sequencing tools (Pyrosequencing and Roche Applied Sciences and 454 Life Sciences GS20 Platforms).

Data source

The metagenomes used in this article are freely available from the SEED platform and are being made accessible from CAMERA and the NCBI Short Read Archive. The NCBI genome project IDs used in this study are: 4441143- 44, 4441148, 4441152-53, 4441579 -86, 4441589, 4441591, 4441595-97, 4441600-02, 4441605, 4441613, 4441618, 4441658-60, 4441662, 4440361-65, 4443688-89, 4443691, 4443693, 4443702-04, 4443706-09, 4443711-15, 4443718-22, 4443724-25, 4441041, 4441056-57, 4441062, 4441590, 4443679-81, 4443683-85, 4443687, 4440411, 4440413, 4440422, 4440440, 4441092, 4441093, 4440453-54, 4440461-63, 4440595, 4440610-11, 4440613-16, 4440639-40, 4440823-26, 4440939, 4440940-51, 4441050,4441599,4440324,4440329,4440416,4440419,4440425-26, 4440429-30, 4440433-35, 4440437-38, 4440963-72, 4441051,4441055,4441057,4441125-30, 4441134-36, 4441139,4441145-47, 4441149-51, 4441155-56, 4441570,4441573-78, 4441587-88,4441592,4441594,4441607,4441609-11,4441614-16,4441661,4443740,4441121,4441133,4441139,4441167,4441593, 4441603-04, 4441617, 4442642-43,4442643, 4442647-53, 4440037, 4440039-41, 4442583, 4443746-47, 4443749-50,4443750,4443762,4441679-84, 4440463-64,4440464,4440056. These processed dataset were collected from the website of Dinsdale Lab., San Diego State University (https://dinsdalelab.sdsu.edu/metag.stats/) published in 2009. A number of statistical techniques were applied to these metagenomics data to explore the relevant phenomenon (Dinsdale et al. 2013).

While various environmental measurements were collected at the time of metagenome sampling, the two data types: environmental and genomic have been analyzed simultaneously to provide direct evidence of how microbial communities differ across environmental gradients. Therefore our analyses used the percent of sequences in each metabolic or functional group as the datasets. The metabolic group is the response variables and the metagenomes were considered as the observations. The 27 functional hierarchies used in the analysis were: amino acids and derivatives; carbohydrates; cell division and cell cycle; cell wall and capsule; cofactors, vitamins, prosthetic groups and pigments; DNA metabolism; dormancy and sporulation; fatty acids, lipids, and isoprenoids; membrane transport; metabolism of aromatic compounds; miscellaneous; motility and chemotaxis; nitrogen metabolism; nucleosides and nucleotides; phages, prophages and transposable elements; phosphorus metabolism; photosynthesis; plasmids; potassium metabolism; protein metabolism; regulation and cell signaling; respiration; RNA metabolism; secondary metabolism; stress response; sulfur metabolism and virulence as classified by the concerned researchers (Aziz et al. 2008).

Statistical and Graphical methods

The data consisted of 10 different types (the environments), 27 response variables (the functional metabolic groups), and 212 observations (the metagenomes). we attempted to analyze multivariate data of the metagenomes using three different widely used statistical techniques namely random forests (RF), multidimensional scaling and linear discriminant analysis with a view to visualize the differences between and within environments and identify the key metabolic processes that might be crucial in the biological process.

Random forests

The random forest (Brieiman 2001) is a robust analytical tool. It is typically used to classify data either in supervised or unsupervised manner. It is a rapid classification technique that is less susceptible to over-fitting data and can be run in a bootstrap fashion (Dinsdale et al. 2013). In addition, the random forest provides a measure of the importance of each variable that can be used in other analyses. There are several approaches that work in conjunction with random forests to estimates the importance of variables in separating the data into groups. One uses the mean decrease in accuracy that a variable causes is determined during the OOB (out-of-bag) error calculation phase. The values of a particular variable are randomly permuted among the set of OOB metagenomes. Then the OOB error is computed again. The more the accuracy of the random forest decreases due to the permutation of values of this variable, the more important the variable is deemed. The mean decrease in Gini is a measure of how a variable contributes to the homogeneity of nodes and leaves in the Random Forest (Dinsdale et al. 2013). Let p_{mgi} be the proportion of samples of group g_i in node m. Let g_c be the most plural group in node m. The Gini index of node ${}^{m}G_m$ is defined in the following equation (i)

$$G_m = \sum_{i \in g} p^2_{mgi} \quad \text{---------- (1)}$$

The Gini index is a measure of the purity of the node, with smaller values indicating a purer node and thus a lesser likelihood of misclassification (Brieiman et al. 2001). Tree generating algorithms may use this index as their likelihood to pick which variable to split on. Each time a particular variable is used to split a node, the Gini indexes for the child nodes are calculated and compared to that of the original node. When node m is split into m_r and m_l, there is a probability p_{m_r} of samples going into the child node m_r and p_{m_l} of going into m_l. The decrease (Brieiman et al. 2001) in Gini is defined in Equation (2)

$$D_m = G_m - p_{m_r} G_{mr} - p_{m_l} G_{p_l} \quad \text{---------- (2)}$$

The calculated decrease is added to the mean decrease Gini for the splitting variable and normalized at the end. The greater the mean decrease Gini of a variable, the purer the nodes splitting.

Each time a particular variable is used to split a node, the Gini coefficients for the child nodes are calculated and compared to that of the original node. The Gini coefficient is a measure of homogeneity from 0 (homogenous) to 1 (heterogeneous). The decreases in Gini are summed for each variable and normalized at the end of the calculation. Variables that split nodes into nodes with higher purity have a higher decrease in Gini coefficient.

Multidimensional scaling

Multidimensional scaling is a data visualization technique that directly scales objects based on either similarity or dissimilarity matrices (Quinn and Keough 2002). MDS takes for its input an n × n dissimilarity matrix S for n metagenomes, constructed by some other statistical technique, such as random forest. Then the algorithm looks for an embedding of the data points into some lower dimensional space that preserves the dissimilarity distances as much as possible. This embedding can then be plotted to visualize the clusters and their distances.

Linear discriminant analysis

Linear discriminant analysis is a robust supervised statistical technique that aims to separate the data into groups based on hyper planes and describe the differences between groups by a linear classification criterion that identifies decision boundaries between groups (Fisher 1936). Let X be a dataset with defined groups 1.....n. For each group j, there exists a corresponding conditional distribution describe in equation (3).

$$X(j) \square G(i) = j - f_j \text{ ---------- (3)}$$

Furthermore, let π_j represent the proportion of X that is contained in group j. To perform a LDA on X, we assume that each f_j is normally distributed with an equal covariance matrix Σ, but with possibly different means μ_j. Using maximum likelihood estimation theory, the linear discriminant functions can be derived in equation (4).

$$g_j(x) = \log(\pi_j) + x\sum^{-1}\mu_j^T - \frac{1}{2}\mu_j\sum^{-1}\mu_j^T \text{ ---------- (4)}$$

These g_j's from (4) are our classifying functions. Since for a point x we sought to maximize $\pi_j f_j$, our classification criterion is

assign x to group j if $g_j(x) > g_k(x)$ for all $k \neq j$

With the classification criterion, decision boundaries between groups can be found. The decision boundaries are where the discriminant functions intersect. That is, the decision boundary between groups j and k is $\{x: g_j(x) = g_k(x)\}$. Therefore, the linear discriminant functions split the data space into regions. Each region corresponds to a specific group and the decision boundaries separate the regions.

Statistical software

The statistical and graphical methods discussed here are implemented using open source Statistical Language Programming R 3.2.2 (www.r-project.org).

Results and Discussion

RF generates a measure of the importance of each variable calculated by either the mean decrease in accuracy or the mean decrease in the Gini. These two values indicate which variables contributed the most to generating strong trees and can be used in MDS and LDA analyses. A subset of the data and variables is used to generate the trees and thus the approach can predict the environment to which a metagenome belongs. For both Accuracy and Gini in Fig.1 and Table 1, the photosynthesis got highest position with score 70.20 and 16.07 respectively as well as the phage groups with second highest score 61.31 and 12.14 for both procedures were the most important response variables in separating the datasets, and in the both cases a break occurred between these two variables and the remaining variables, suggesting that just these two measures could be used to grossly classify the metagenomes. Eight variables with highest Mean Decrease Accuracy and Mean Decrease Gini score were thus chosen for the following MDS and LDA analyses.

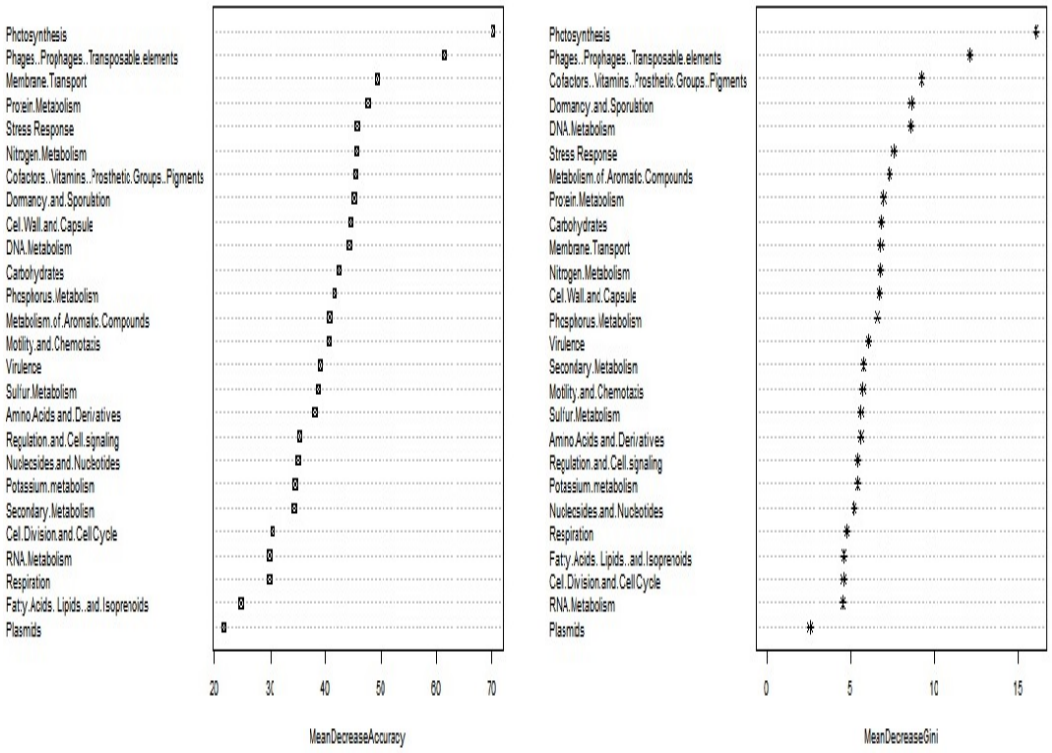

Fig. 1. Variable importance determined by random forest analysis using mean decrease in Accuracy and Gini. The plot outcome measures ranks of 26 the metabolic functions correspond to each symbols in both plots are placed according to their importance score in descending order.

Table 1. Variable importance measure with corresponding score.

Metabolic variables	Mean decrease in accuracy	Mean decrease in Gini	Metabolic variables	Mean decrease in accuracy	Mean decrease in Gini
Amino acids and derivatives	38.11	5.62	Phages, Prophages, and Transposable Elements	61.31	12.14
Carbohydrates	42.49	6.83	Phosphorus Metabolism	41.59	6.58
Cell Division and cell cycle	30.49	4.55	Photosynthesis	70.20	16.07
Cell wall and capsule	44.73	6.72	Plasmids	21.66	2.52
Cofactors, vitamins, Prosthetic groups, and pigments	45.49	9.2	Potassium Metabolism	34.54	5.40
DNA metabolism	44.44	8.58	Protein Metabolism	47.65	6.97
Dormancy and sporulation	45.29	8.61	Regulation and Cell Signaling	35.28	5.41
Fatty acids, lipids, and isoprenoids	24.84	4.57	Respiration	29.81	4.75
Membrane Transport	49.29	6.80	RNA Metabolism	29.82	4.52
Metabolism of Aromatic Compounds	40.68	7.34	Secondary Metabolism	34.40	5.76
Motility and Chemotaxis	40.56	5.73	Stress Response	45.79	7.60
Nitrogen Metabolism	45.68	6.76	Sulfur Metabolism	38.76	5.61
Nucleosides and Nucleotides	35.08	5.19	Virulence	39.11	6.05

MDS projects the proximity measures of the metagenomes as determined by RF to a lower-dimensional space (e.g., 2-dimensional space for plotting on xy-axis). For the RF, the similarity was measured as the number of times two metagenomes appeared on the same leaf in the trees (proximity), and is represented by the distance between two samples on the MDS plot. The MDS plots have been shown in Fig. 2 with the 10 predefined environments. In this analysis, the visualization highlights the separation of the microbes from human/animal hosts from other samples along the first dimension and the separation of the aquatic and mat communities along the second dimension.

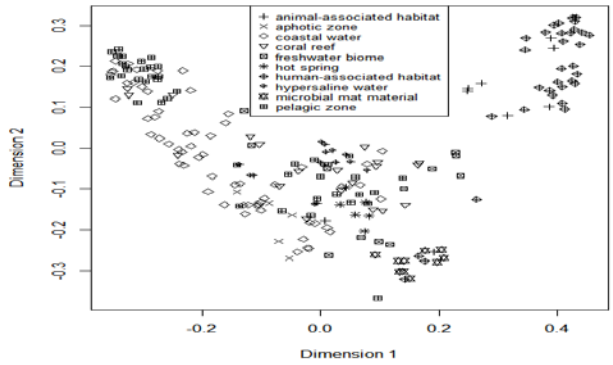

Fig. 2. Multiple dimensional scale plots of the distances calculated from unsupervised random forest. The distances are the number of times the samples appear on the same leaf of the tree, and the MDS has scaled them so that they plot projects those distances into two dimensions. Plotted by the original environments the sample came from.

In Fig. 3 the LDA overall 27 metabolic variables separated the data and showed that the human and terrestrial associated animal metagenomes separated from a cluster consisting of all of the aquatic samples except the hyper-saline community. The mat samples separated distinctly from other cluster

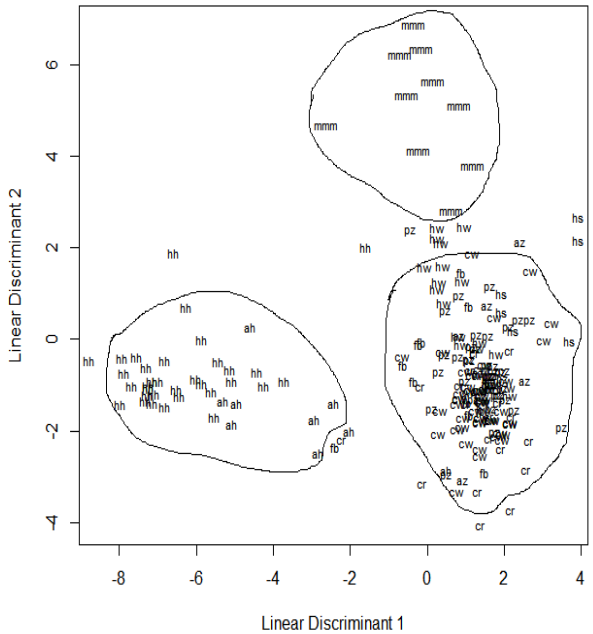

Fig. 3. Linear discriminate analysis showing the position of the metagenomes in two-dimensional space from the 10 environments.

For more detail and effective statistical analyses in terms of classification of the metagenomes correspond to their environments from which they belonged and sequenced, the robust statistical approaches should be

employed. DNA sequence count of metagenomes may however suffer from the presence of extreme values. This kind of characteristics of the data increases the misclassification error rate and as a result provides low accuracy and precision of the statistical analyses. The robust or noble statistical or classification techniques will simultaneously deal the fact of presence of extreme as well as missing values in the DNA count dataset of metagenomes and provide decent appropriate explanatory and conclusive results.

Conclusion

The analyses separated the microbial samples into three broad groups: the human and animal associated samples, the microbial mats and the aquatic samples. The RF technique showed that phage activity is a major separator of host-associated microbial communities and free-living, suggesting that the phages are playing different ecological roles within each environment. The MDS and LDA techniques suggest that mat communities separated from both the animal associated metagenomes and the aquatic samples by the vitamin and cofactor metabolism, suggesting a role for secondary metabolism associated with growth in extreme environments. The dominant metabolic feature that separated the aquatic samples was photosynthesis. The marine environment categories of open ocean, coastal waters, coral reef and deep oceans share many metabolic features and therefore these metagenomes were placed into categories different than their a priori group assignment. This suggests subtle variation in metabolic processes that are occurring in the microbial communities from each environment that should be investigated in the future.

Acknowledgement

Authors would like to acknowledge to Bangladesh Agricultural Research Institute (BARI) for granting leave and Bioinformatics Lab., Department of Statistics, University of Rajshahi, Bangladesh for financial support for this study.

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J. Bio-Sci. 24: 55-65, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

IN SILICO ANALYSES OF HUMAN COLLAGEN PROTEIN FUNCTION PREDICTION

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Abstract

Collagen is the extracellular matrix protein in the several connective tissues in the human body. It is an important component for mediating cell-cell interactions and pathological conditions in human body. In this study we perform the analysis of physiochemical properties and investigate the functional characteristics of human collagen proteins. Also investigate the functional protein groups by the statistical analysis. The collagen protein family consisting 28 members in human which are involving in the complex structure of protein. The protein function, protein sequence properties, domain composition, phylogenetic and protein-protein interaction (PPI) networks analysis of human collagen alpha-1 protein sequences are implemented by the online bioinformatics tools which are currently available. Based on the PCA analysis amino acid composition, features of collagen protein sequences are divided into two supreme influential functional groups such as collagen 12, 14, 20 formed one group and the rest of others formed another group. The protein-protein interaction network study using STRING showed that top interacting score of functional group proteins 0.952, 0.939 and 0.929. The most common functional domain of collagen proteins are VWC, C4, LamG, VWA, KU, C1Q, TSPN and FN3. Physicochemical, functional and phylogenetic classification can give extensive information of protein's structure and function. The depiction of alpha-1 chains of collagen protein family in human collagen 12, 14 and 20 as a prospective protein cluster. These three proteins are possess, low glycine and proline, very high aliphatic index and a close evolutionary relation in the human skin.

Key words: Collagen protein sequences, k-means clustering, PCA, phylogenetic tree, PPI network, protein domain structure

Introduction

The extracellular matrix (ECM) is consisting of collagens, proteoglycans, glycoproteins and proteases. The extracellular matrix of connective tissues represents a complex alloy of variable members of diverse protein families defining structural integrity and various physiological functions. It is the main component of connective tissue and makes up from 25% to 35% of the whole-body protein content. Collagen Type I protein found in bone, skin, muscles and walls of blood vessels in human body (Järveläinen et al. 2009). Neighboring a substantial volume of cells the ECM is an intricate network of macromolecules. For the multiple processes such as cell migration, cell-cell interaction and cell proliferation these components play vital role (Bowers et al. 2010). The collagen protein is a triple helical structure of polypeptide chains, commonly known as the alpha chains. The common sequence pattern of triple helix is "Gly-X-Y" (Kadler et al. 1996). The stability of the helical structure depends on the presence of glycine as every third residue and being other property of the smallest amino acid. Any amino acid can be taken instead of X and Y but frequently occupied by the proline residue. Every mature active collagen protein molecules were shown that the peptidases form of pro-peptides present at the N and C terminal. The genetically distinct 28 members

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found in human biological system. The collagen family is a large and a complex protein family in the proteome (Gelsea et al. 2003). The genomics, proteomics and the computational biology is an evolving field that helps to understand the concealed information of a protein structure (Nassa et al. 2012). In this study reports a qualified portrayals of alpha-1 sequences of human collagen using statistical methods and bio-computational tools. Three alpha chains present in collagen alpha-1 was pragmatic human collagen family. To analysis of physiochemical properties, functional group, phylogenetic classification, domain composition and PPI networks of human collagen using the amino acid sequence features. Objective of this research is to afford an intuition to various proteins features of collagen proteins and represent this large family. The presence of any nonconforming entrances in the collagen molecules for impending adherents to implicate the disease in collagen family (Bateman et al. 2009). Most of the previous studies are based on the wet lab experimental and it is very much time consuming, costly and laborious for identification of functional collagen proteins in the human skin. In this case we have suggested some *in silico* and statistical methods for functional analysis of collagen proteins. It might be reduced time and cost in comparison with experimental methods. This study might be helpful for biologists in stentorian of promote soundings on these complex molecules, interacting proteins, and functional domains for human diseases in collagen family.

Materials and Methods

Collection of human collagen apha-1 sequences

Human collagen family alpha-1 protein sequences of all 28 members were collected in FASTA format using the accession number provided for each collagen sequence from UniProtKB/ SWISS-PROT (http://expasy.org/sprot/) (Bairoch et al. 2000) protein database.

Physiochemical Portrayal of Human Collagen Family

Various features including the number of amino acids, molecular weight, theoretical isoelectric point (pI), amino acid composition (%), number of positively (Arg + Lys) and negatively charged (Asp + Glu) residues, extinction co-efficient, instability index, aliphatic index and Grand Average of Hydropathicity (GRAVY) were computed using ExPASy's ProtParam tool by inputting the protein sequence in FASTA format (http://expasy.org/tools/protparam.html).

Protein domain composition of human collagen family

The protein domain composition and post translation sites prediction using SMART (http://smart.embl-heidelberg.de/) (Letunic et al. 2012) bioinformatics tool also used to scan and identify all the known domain. To predict the nature and position in the selected alpha-1 protein sequences of the collagen family based on a profile and pattern search. The input protein sequence in FASTA format was used for a selected protein profile in the database.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of the human alpha-1 collagen protein sequences were aligned using MEGA5.0 tools (Tamura et al. 2011), the sequence alignment algorithm was used ClustalW of protein sequences in FASTA format as the input data type. For a set of input sequences the best alignment was computed and all the identities. The phylogenetic tree or evolutionary tree was customary by constructing phylograms through recovery of the alignments using Neighbor Joining (NJ) method.

Protein-protein interaction (PPI) networks analysis

The accurate prediction of protein functions is important for interacting residues with each other. This study used STRING (http://string-db.org/) a database of known and predicted protein interactions networks through physical and functional associations (Andrea et al. 2013). The input protein sequence was used in FASTA format for prediction of PPI networks.

Statistical analysis of collagen protein sequences

The analysis of amino acid functional group was used k-means clustering approach. To investigate the collagen protein functional group we used the multivariate statistical techniques principal component analysis (PCA), it is very much popular techniques in bioinformatics data analysis. In this paper, all the statistical analysis likes k-means clustering and PCA were done using R-packages(R 3.2.0) and MS Excel-2010.

Results and Discussion

The portrayals of human collagen alpha-1 extracellular matrix protein are most important for human skin. Collagen protein sequence physiochemical properties analysis was done by the ExPASy ProtParam online tools (Table 1). The highest aliphatic index is 79.61 of Col20 (Fig. 1a) was regarded as the thermostable and Col14 (77.67) and Col12 (75.45). The GRAVY values (Fig.1 b) indicate the range from -2 to +2 of proteins are positively related with the proteins being more hydrophobic (Kyteet al. 1982).

Table 1. Physiochemical properties of collagen protein family.

Collagen Proteins	Accession number	No. of AA	Molecular weight	pI	-ve charged residue	+ve charged residue	Extinction Coefficient	Instability index	Aliphatic index	GRAVY
Coll 1	P02452	1464	138941.5	5.6	141	128	53495	30.43	37.98	-0.788
Coll 2	P02458	1487	141785.3	6.58	141	139	54525	25.21	40.03	-0.803
Coll 3	P02461	1466	138564.2	6.21	129	122	62225	30.18	37.31	-0.797
Coll 4	P02462	1669	1606147.7	8.55	128	138	61070	32.04	47.39	-0.621
Coll 5	P20908	1838	183559.8	4.94	225	168	98850	33.09	45.35	-0.873
Coll 6	P12109	1028	108529.4	5.26	139	114	64970	28.52	68.70	-0.525
Coll 7	Q02388	2944	295219.6	5.95	332	310	159140	32.07	61.86	-0.625
Coll 8	P27658	744	73364	9.62	37	60	38405	36.06	61.21	-0.434
Coll 9	P20849	921	91869.2	8.94	86	96	42565	32.61	56.13	-0.658
Coll 10	Q03692	680	66157.9	9.68	34	54	42290	25.95	51.94	-0.556
Coll 11	P12107	1806	181064.8	5.06	222	174	103765	30.81	44.91	-0.859
Coll 12	Q99715	3063	333146.7	5.38	366	313	334620	32.90	75.45	-0.427
Coll 13	Q5TAT6	717	69949.9	9.27	67	81	15970	31.44	52.87	-0.765
Coll 14	Q05707	1796	193515.4	5.16	211	160	179095	37.57	77.67	-0.326
Coll 15	P39059	1388	141720.1	4.90	155	95	76485	40.19	68.00	-0.377
Coll 16	Q07092	1604	157751.3	8.14	144	150	65370	35.88	50.73	-0.671
Coll 17	Q9UMD9	1497	150419.3	8.89	117	128	109015	45.25	55.47	-0.573
Coll 18	P39060	1754	178187.6	5.67	164	133	145185	48.57	61.72	-0.467
Coll 19	Q14993	1142	115220.7	8.57	116	124	63215	30.68	56.68	-0.708
Coll 20	Q9P218	1284	135830	8.27	119	123	132990	45.18	79.61	-0.261
Coll 21	Q96P44	957	99368.5	8.57	98	106	55655	33.28	69.14	-0.517
Coll 22	Q8NFW1	1626	161145.3	6.88	174	172	57965	34.00	53.28	-0.715
Coll 23	Q86Y22	540	51943.9	6.88	65	65	14355	30.81	50.69	-0.829
Coll 24	Q17RW2	1714	175496.3	8.46	162	170	73075	28.32	64.21	-0.622
Coll 25	Q9BXS0	654	64770.7	8.60	73	78	11835	24.85	47.19	-0.919
Coll 26	Q96A83	441	45381.1	7.02	40	40	40170	46.77	63.11	-0.523
Coll 27	Q8IZC6	1860	186892.3	9.83	136	196	81205	37.62	54.15	-0.637
Coll 28	Q2UY09	1125	116657.1	6.10	136	131	55195	24.18	61.42	-0.66

The collagen 20 GRAVY is -0.261 then we may state that it is most hydrophobic protein than others. From the table collagen 15, 17, 18, 20 and 26 are unstable (instability index >40) and rest of the proteins are stable (instability index <40), the all values of instability index shows in Fig. 1(c).The 14 collagens pI (Fig. 1d) are less than 7, Col26 is approximate equal to 7 and rest of the greater than 7; hence the 14 collagens are acidic, Col26 is neutral and others collagen proteins are basic (Lim 2006).

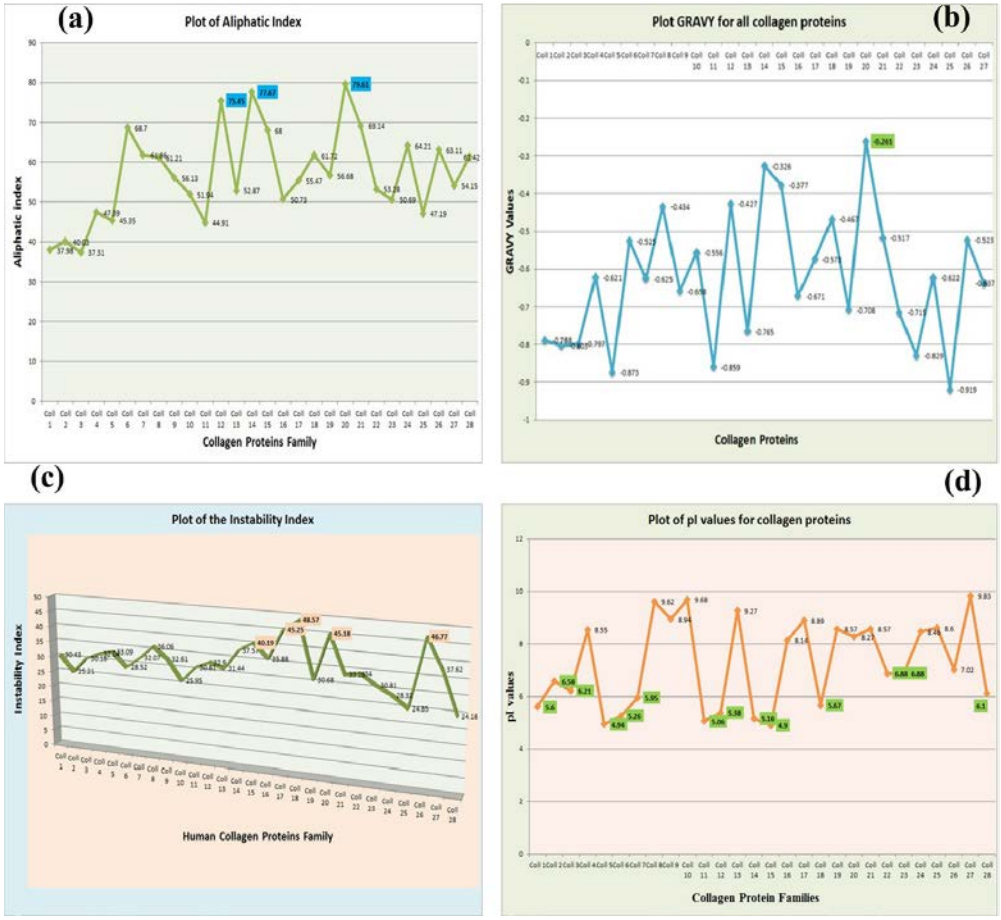

Fig. 1. (a) Aliphatic index, (b) GRAVY values, (c) Instability index and (d) pI values for all 28 human collagen alpha-1 families.

The common domain structure is COLFI (C-termini of Fibrillar collagen) of Coll1, Coll2, Coll3, Coll5, Coll11, Coll14 (Fig. 2) and the other domains are VWC (Von Willebrand factor type C), C4 (C-terminal tandem repeated), LamG (Laminin G), VWA (von Willebrand factor type A), FN3 (Fibronectin type-III), C1Q, TSPN (Thrombospondin N-terminal), FRI (Frizzled) and KU (BPTI/Kunitz family of serine protease inhibitors). From the Table 2 shown that Coll13, Coll15, Coll23, Coll25 and Coll26 proteins has no functional domain out of 28 human collagen protein families in the human skin. The maximum number of domains exists in the Coll12 (E-values: 1.28e-8 to 1.25E-78), Coll14 (E-values: 4.22e-9 to 0.214) and Coll20 (E-values: 19.6e-55 to 2.89e-33) proteins, those three protein domains are more functional activity of the ECM proteins.

Table 2. Human collagen alpha-1 protein domains.

Collagen Proteins	Source Gene	Domain Name	Start	End	E-value
Col 1	ENSG00000108821	VWC	40	95	2.73E-20
		COLFI	1228	1464	1.25E-166
Col 2	ENSG00000139219	VWC	34	89	7.42E-22
		COLFI	1252	1487	6.46E-183
Col 3	ENSG00000168542	VWC	32	88	1.68E-20
		COLFI	1231	1466	1.15E-165
Col 4	ENSG00000187498	C4	1445	1554	3.55E-66
		C4	1555	1668	3.70E-78
Col 5	ENSG00000130635	TSPN	39	230	6.53E-82
		LamG	98	229	6.50E-04
		COLFI	1608	1837	1.06E-155
Col 6	ENSG00000142156	VWA	35	233	4.29E-31
		VWA	613	790	8.00E-31
		VWA	827	1008	2.72E-33
Col 7	ENSG00000114270	VWA	36	216	4.54E-53
		FN3	232	318	5.73E-11
		FN3	327	402	6.54E-06
		FN3	415	493	9.11E-05
		FN3	508	584	1.64E-06
		FN3	598	674	1.94E-08
		FN3	686	764	1.16E-11
		FN3	776	853	6.35E-04
		FN3	867	943	7.45E-10
		FN3	955	1039	5.04E-07
Col 8	ENSG00000144810	C1Q	609	744	3.27E-79
Col 9	ENSG00000112280	TSPN	50	244	2.02E-87
Col 10	ENSG00000123500	C1Q	545	680	6.20E-80
Col 11	ENSG00000060718	TSPN	38	229	1.15E-68
		LamG	97	228	9.48E-06
		COLFI	1576	1805	7.39E-128
Col 12	ENSG00000111799	FN3	25	103	1.28E-08
		VWA	138	317	1.21E-62
		FN3	334	413	1.35E-07
		VWA	438	617	5.62E-58
		FN3	632	710	2.16E-06
		FN3	723	801	1.74E-10
		FN3	814	892	6.59E-11
		FN3	905	984	2.23E-08
		FN3	995	1074	9.54E-08
		FN3	1087	1166	4.09E-07
		VWA	1197	1376	3.46E-58
		FN3	1385	1463	2.46E-10
		FN3	1474	1554	3.29E-11
		FN3	1566	1643	9.83E-10
		FN3	1655	1734	7.63E-07
		FN3	1753	1832	1.09E-11
		FN3	1844	1922	4.09E-07
		FN3	1934	2013	9.69E-09
		FN3	2025	2104	3.73E-10
		FN3	2116	2193	7.57E-11
		FN3	2204	2283	6.35E-04
		VWA	2321	2501	7.09E-55
		TSPN	2520	2712	1.25E-78

Table 2 Contd.

Col 13	ENSG00000197467	-	-	-	-
Col 14	ENSG00000187955	FN3	30	108	4.22E-09
		VWA	156	335	3.36E-56
		FN3	353	433	3.32E-07
		FN3	443	521	6.20E-07
		FN3	535	612	8.83E-12
		FN3	624	703	4.77E-08
		VWA	1030	1210	7.53E-59
		TSPN	1229	1424	2.46E-68
		FN3	735	817	9.25E-06
		FN3	829	908	1.45E-07
		FN3	919	998	2.14E-01
Col 15	ENSG00000204291	-	-	-	-
Col 16	ENSG00000084636	TSPN	50	231	3.82E-74
Col 17	ENSG00000065618	-	-	-	-
Col 18	ENSG00000182871	FRI	333	448	1.25E-25
		TSPN	456	644	6.32E-57
		LamG	505	643	1.11E-01
Col 19	ENSG00000082293	TSPN	50	234	1.01E-73
Col 20	ENSG00000101203	FN3	26	102	1.96E-54
		VWA	177	356	7.66E-51
		FN3	377	457	1.57E-08
		FN3	466	546	1.13E-09
		FN3	557	636	1.55E-07
		FN3	647	726	2.72E-03
		FN3	741	820	4.12E-12
		TSPN	842	1037	2.89E-33
Col 21	ENSG00000124749	VWA	35	212	3.02E-49
		TSPN	230	412	2.18E-19
Col 22	ENSG00000169436	VWA	36	218	3.83E-51
		TSPN	239	427	1.55E-33
Col 23	ENSG00000050767	-	-	-	-
Col 24	ENSG00000171502	TSPN	68	228	1.02E-05
		COLFI	1514	1714	3.85E-35
Col 25	ENSG00000188517	-	-	-	-
Col 26	ENSG00000160963	-	-	-	-
Col 27	ENSG00000196739	TSPN	45	222	1.46E-05
		OLFI	1659	1860	1.41E-42
Col 28	ENSG00000215018	VWA	46	228	3.06E-18
		VWA	796	973	3.02E-40
		KU	1070	1123	1.08E-19

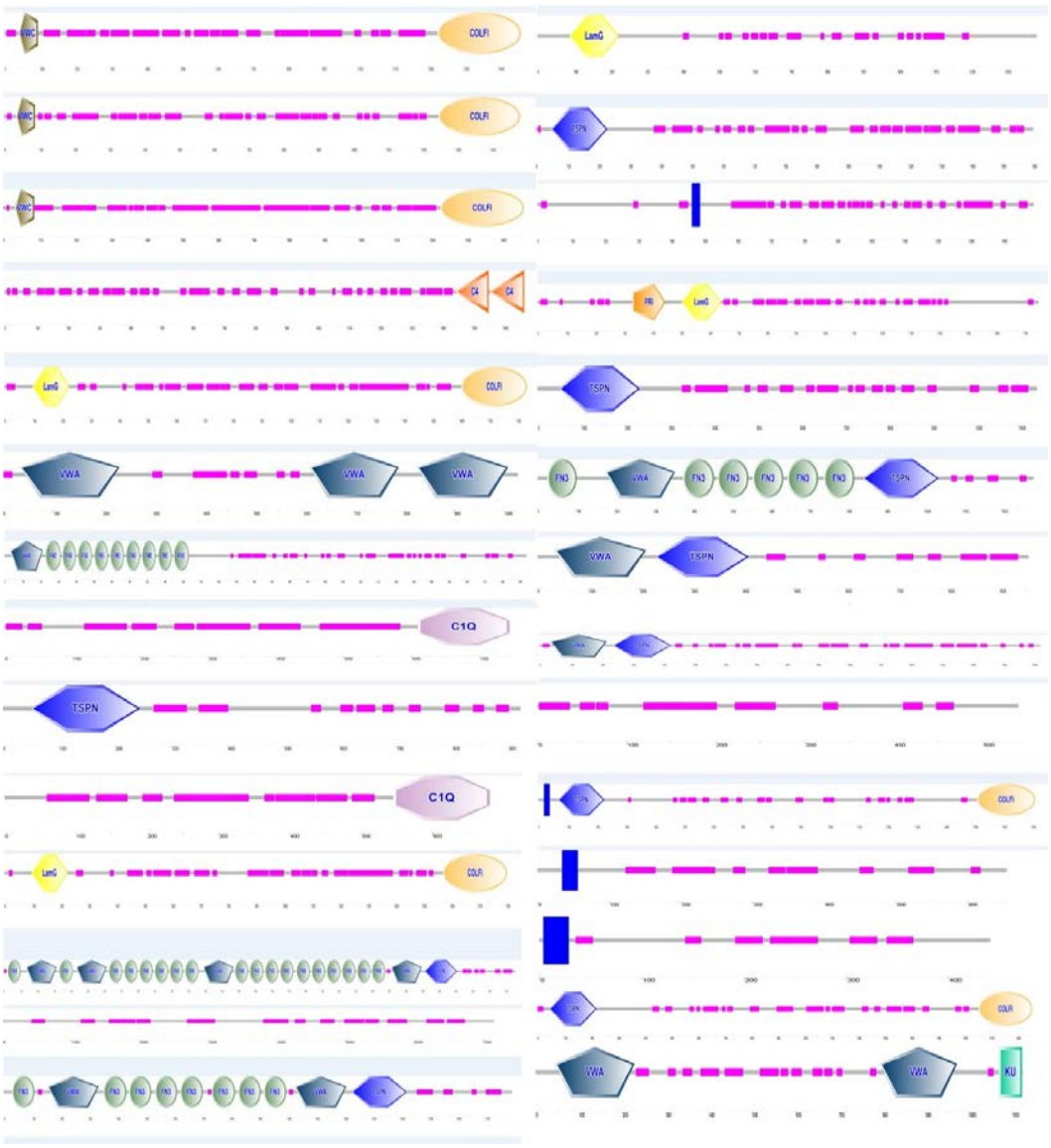

Fig. 2. Protein domain structure of 28 human collagen alpha-1 families.

For the evolutionary study, the phylogenetic analysis shows (Fig. 3a) shows four group of collagen proteins in the 28 family members; the CollXXV and CollXXIII (bright pink), CollXX, CollXII, CollIV (bright green), CollXXVI (red) and rest of the collagen are same groups (blue). In the blue collagen group, there are two different sub-groups CollVI (green) and CollXXVIII (maroon). Hence the CollXXVI is completely separate from the other functional protein groups. Clustering is the unsupervised techniques in machine learning approaches to cluster different groups based on the within groups similarity and between groups dissimilarity.

For the k-means clustering approach first important issues is the selection of number of k; there are several methods exists in the literature for selecting the optimum number of k. Scree plot one of them popular methods for selecting k (Fig. 3b), it was shown two (k = 2) optimum clusters for clustering the amino acid properties. By the k-means clustering shown that in different k = 2, k = 3, k = 4 (Fig. 3c) than the finest two functional properties in the amino acid i.e. positively charged and negatively charged. The principal component analysis (PCA) based clustering approach is the modern multivariate techniques. In this paper we used this techniques for identify the joint functional protein complex in the human collagen protein families (Fig. 3d) using biplot; the standardized PC1 is explained 90.6% and standardized PC2 is 4.9% with compare the total features. Therefore, the two functional protein complexes are in the human collagen alpha-1 proteins. The collagen 12, 14 and 20 are similar protein complex shows the similar properties and rest of the collagens is others group.

The protein-protein interaction network study investigate the jointly and similar functional activity based on the interacting score. The PPI networks analysis (Fig. 4) for identifying the most interacting functional collagen protein groups of 28 human collagen families was done using the STRING database. The top interacting score of Coll12, Coll14 and Coll20 proteins are 0.952, 0.939 and 0.929 respectively (Fig. 5).

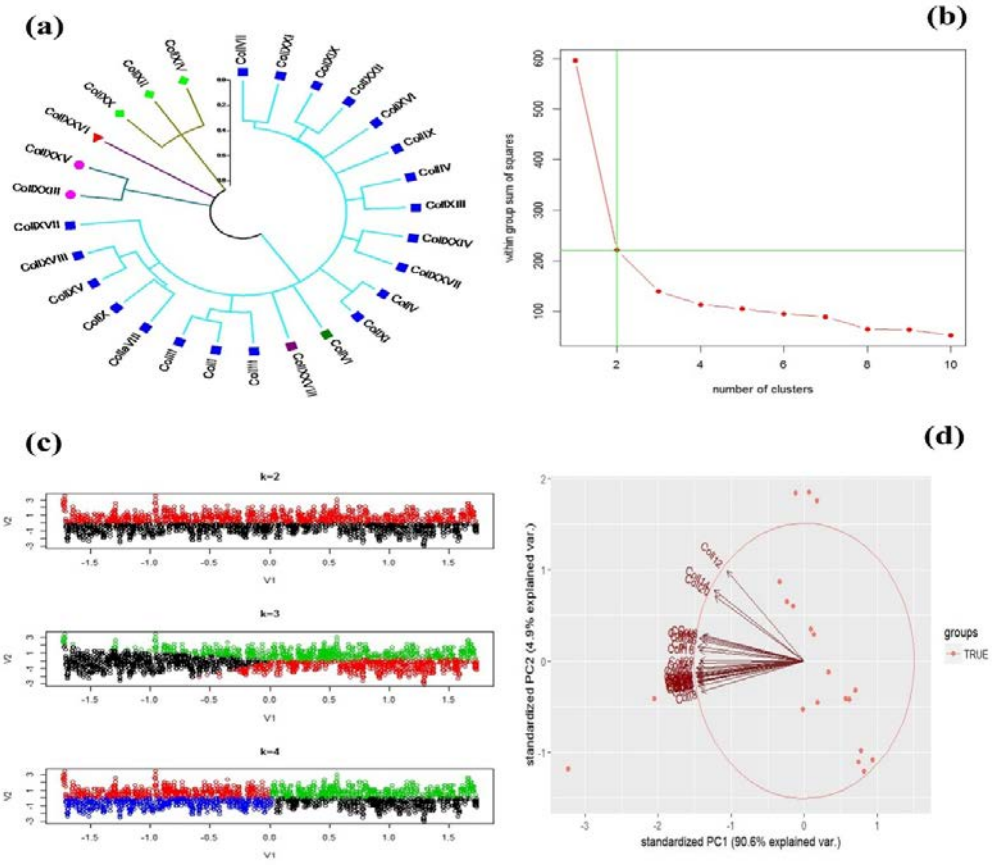

Fig. 3. (a) Phylogenetic tree, (b) Scree plot, (c) K-means clustering and (d) Biplot for the analysis of human collagen family (28) of alpha-1.

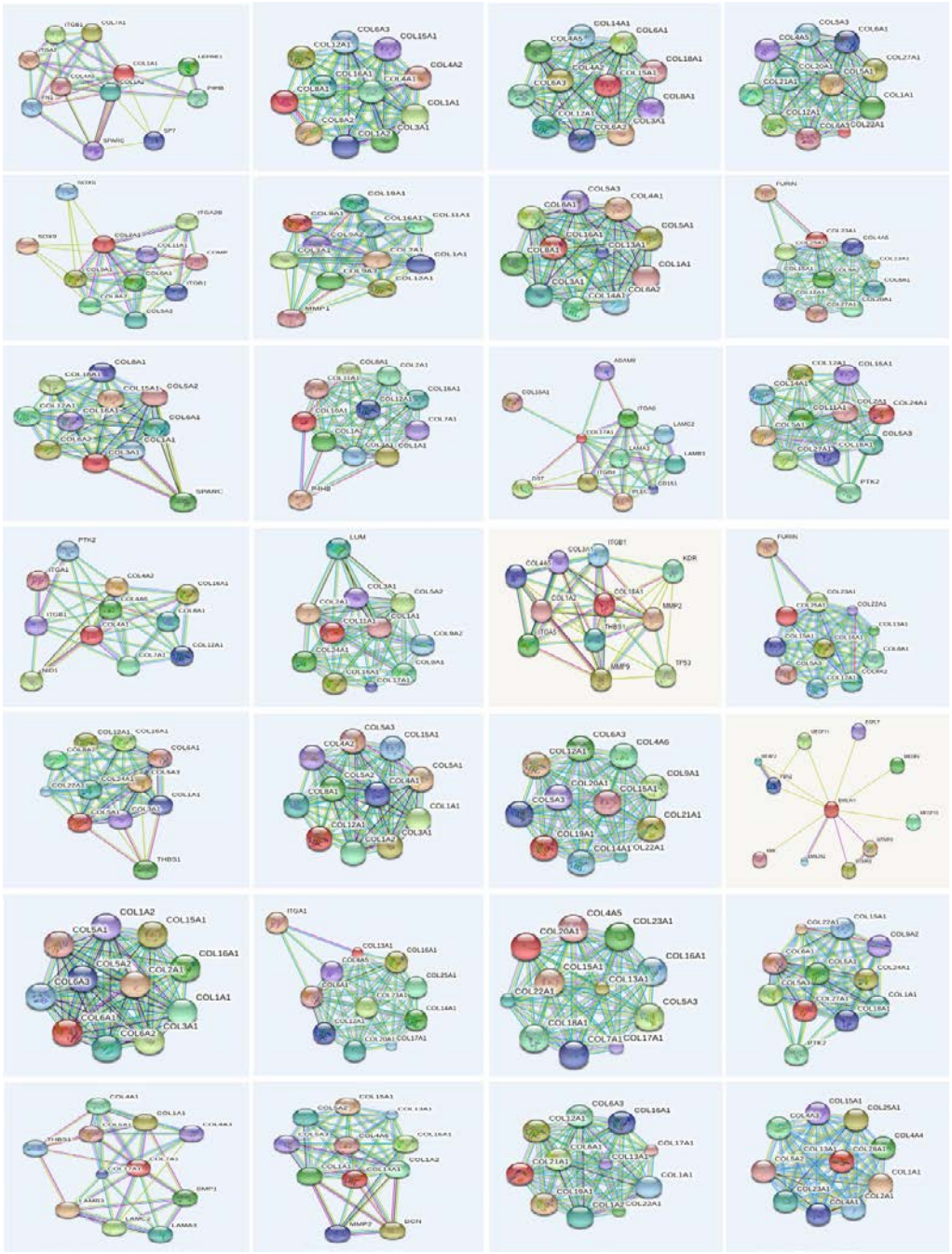

Fig. 4. Protein-protein interaction (PPI) network for finding the most similar functional interacting proteins by the STRING data base of 28 human collagen families.

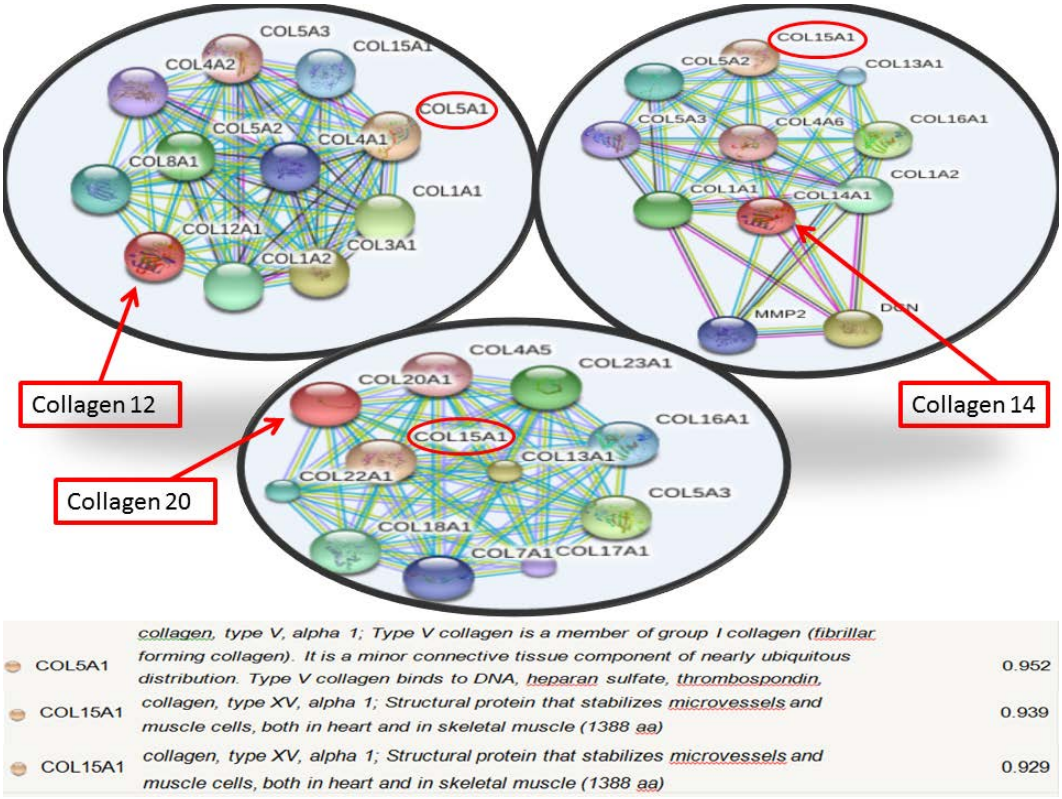

Fig. 5. Protein-protein interaction network for most influential functional interacting group proteins by the STRING data base of Coll12, Coll14 and Coll20 based on the human collagen families.

Conclusion

The *in silico* analysis of extracellular matrix proteins is the most important for studying the functional characteristics of human collagen families. The analysis of functional protein domain shows the lots of significant domains are Coll12, Coll14 and Coll20. In this study we used the positively and negatively charged amino acids, that's justify by the screen plot and k-mean clustering approaches. By the principal component analysis approaches it is shown that, the first 2 PC's are explained approximately 95% out of the total variations. The PC's score plot gives us the two most important functional groups, one of them group collagen proteins are collagen 12, 14 and 20 respectively. The above discussion shown that the most important functional collagen proteins are Coll12, Coll14 and Coll20 based on the several analysis tools including statistical techniques. Those collagens are the FACIT (Fibril Associated collagens with Interrupted Triple helices) group of collagen family. This *in silico* study is very much helpful for biologist to analysis of the ECM collagen alpha-1 28 protein families of human skin by the reducing the experimental cost, saving consuming time and laborious work in this field.

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J. Bio-Sci. 24: 67-74, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

INCIDENCE OF SPIRALING WHITEFLIES *ALEURODICUS DISPERSUS* RUSSELL (HEMIPTERA: ALEYRODIDAE) AND ITS NATURAL ENEMIES ON GUAVA ORCHARDS

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Abstract

Population dynamics of spiraling whitefly and its natural enemies on guava orchards are essential for improvement of existing pest control methods. To find out the seasonal abundance of guava whitefly and its natural enemies and levels of infestations were studied on guava orchards severely infested by *A. dispersus*. Five guava plants were selected randomly. To determine each stage of the insect density, six leaves comprising upper, middle and lower part of each plant were randomly sampled. Sooty mould infested leaves and presence of natural enemies of *A. dispersus* were also recorded. The highest number of egg mass (5.0) and nymphs (10.0) per leave were found in the month of September. The highest (0.20%) ladybird beetle, *Scymnus* sp. (Coccinellidae: Coleoptera) and *Encarsia* sp. (0.15%) were found in the months of October and January, respectively. The number of leaves infested with sooty mould fungus was estimated highest (93%) during October to January. *A. dispersus* population was peak in the months of August to January and the associated natural enemies may successfully contribute to the existing pest control methods.

Key words: Guava, infestation, natural enemies, spiraling whitefly

Introduction

The spiraling whitefly, *Aleurodicus dispersus* Russell (Hemiptera: Aleurodidae) is a destructive invasive pest of many crops including guava throughout the world (Martin 1987, Zhang et al. 2004). It has the potential to become widespread and gradually extended its geographical distribution to South Asia, Australia, Africa, Caribbean Islands, Central America and the Indian Ocean (Zhang et al. 2004). Both immature and adults may cause damage to the host plants. The pest sucks plant juices from the phloem through a slender stylet resulting reduced the growth and vitality of host plants. It also excretes honey dew which covers the surface of the leaves and serves as a medium for the growth of sooty mould (Bryne et al. 1990). The sooty mould may also increase thermal absorption and raise leaf temperature, thus reduces leaf efficiency causing premature death of tissue (Bryne et al. 1990, Gungah 2005). *A. dispersus* along with *A. nubilans* (Backton), *A. spiniferus* (Quaintance) and *A. uglami* (Ashby) were reported to be the pest of guava in Bangladesh (Scanlan 1995). A loss of 80% in fruit yield has been recorded in guava attacked by the pest in four continuous months (September to December) in Taiwan (Wen et al. 1995).

Chemical control has widely been used for the management of *A. dispersus*. But all life stages of whiteflies are difficult to control with conventional insecticides because of rapid multiplication, damage under surface of the leaves, thereby not being easily targeted by direct hit of spraying insecticides (Bryne et al. 1990). Spiraling whiteflies were attacked by different natural enemies. Among them the parasitoids *Encarsia guadeloupae* Viggiani and *Encarsia haitiensis* Dozier proved to be highly useful in suppressing the spiraling

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whitefly in Pacific Islands, African and Asian countries (Mani and Krishnamoorthy 2002). Successful introduction of natural enemies is one of the suitable biological control methods to save environment also possible in Bangladesh. Thus, ecological data of spiraling whiteflies and its natural enemies are essential for improvement of existing pest control methods and development of new control strategies in integrated pest management of guava orchards in Bangladesh. So far, the information on the population dynamics of spiraling whitefly and associated natural enemies on guava orchards is scanty especially northern part in Bangladesh. Therefore, the present study was aimed to find out the seasonal abundance of guava whitefly and associated its natural enemies in different guava orchards in northern part of Bangladesh.

Materials and Methods

Experimental site and climatic conditions

The experiment was conducted at Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur from September 2011 to August 2012. The experimental site was located at 25˚ 13' N latitude and 88˚ 23' E longitudes at elevation of 37 m above the sea level. Seasonal abundance of *A. dispersus* and associated its natural enemies were studied in three guava gardens at HSTU. The orchards were separated 50 to 100 meters apart.

The experimental area was under subtropical climate characterized by high temperature, high humidity and heavy precipitation with occasional winds in April to September. But scanty rainfall associated with moderate low temperature during October to March. Data on temperature and relative humidity (RH) were recorded by Hobo data logger (H8-003-02, Onset Computer Corporation, Bourne, MA, USA).

Data collection

Levels of infestations in different months of the year were studied on guava orchards that were severely infested by *A. dispersus*. In each orchard, five guava plants were selected randomly. To determine the number of *A. dispersus,* six leaves comprising two from upper, two from middle and two from lower part of each plant were sampled randomly. From each garden, a total 30 leaves were sampled for and observed insect infestation. The number of egg masses, nymphs, and adults of *A. dispersus* were counting on every sampling day. Sooty mould infested leaves and presence of natural enemies of *A. dispersus* were also recorded. Orchards were free from insecticide applications during the study period.

Statistical analyses

The mean number of egg masses, nymphs, adults and sooty mould infested leaves and their natural enemies in the orchards were analyzed with analysis of variance (ANOVA) and Tukey-type multiple comparison tests for post-hoc analysis (Zar 2010) using SPSS software (version 16.0).

Results and Discussion

Seasonal abundance of *A. dispersus*

The number of egg masses in three guava gardens (Garden A, B and C) recorded in different months is presented in Fig. 1. The results suggested that the highest number of egg masses appeared in the month of November but gradually decreased in different months. Significantly the highest ($F = 10.2$, $df = 2$, $P < 0.001$) number of egg deposition was found in the orchard B. More number of egg masses was produced during the month of September and November which may be considered as the peak period of infestation by *A. dispersus* (Fig. 1).

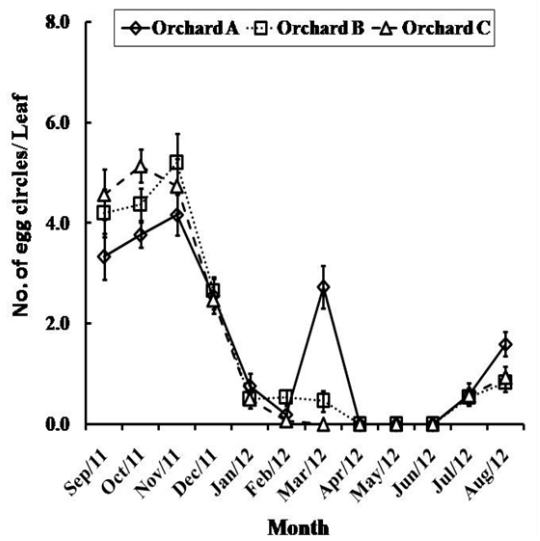

Fig. 1. Seasonal abundance of egg mass of *A. dispersus* on guava leaves in Bangladesh during September 2011 and August 2012.

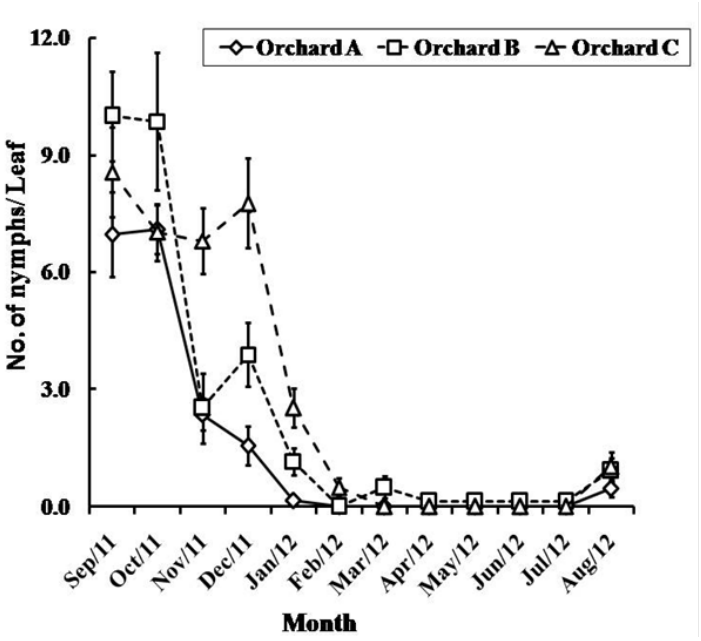

Fig. 2. Seasonal abundance of nymphs of *A. dispersus* on guava leaves in Bangladesh during September 2011 and August 2012.

The number of nymphs in three guava gardens recorded in the different months was shown in Fig. 2. Maximum number of *A. dispersus* nymphs were found in the orchard B. Significantly the highest number of nymphs (F = 16.5, df = 2, P<0.001) were found in garden B which is different from orchard A and C (Fig. 2). The mean number of nymphs per leaf was significantly the highest in the month of September to October (F= 86.21, df = 11, P<0.001) then other month in the year. After January the mean number of nymphs was decreased but no infestation found in the month of February to July.

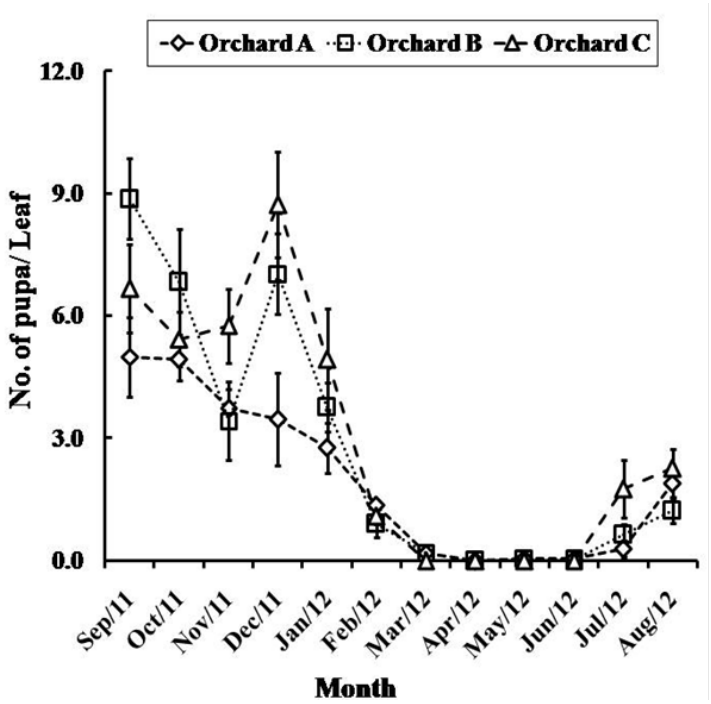

Fig. 3. Seasonal abundance of pupae of *A. dispersus* on guava leaves in Bangladesh during September 2011 and August 2012.

The pupa of spiraling whitefly was highly populated during the month of September (Fig. 3). Increased number of pupae per leaf of guava were found in the orchard B (F = 8.80, df = 2, P <0.001) and significantly different from orchard A and C. Pupae were found significantly highest in the month of September (F = 51.42, df = 11, P <0.001) in all three orchards. But no infestation was found in the month of March to June.

The adult of spiraling whitefly was most densely populated in the month of August (Fig. 4). The highest number of adults per leaf on guava plant were found in the orchard C (F = 19.50, df = 2, P <0.001) and it was statistically different from orchard A and B (Fig. 4). Adult population were increased in the month of August (F = 18.05, df = 11, P <0.001) and continued up to the December but no infestation was found in the month of February to June.

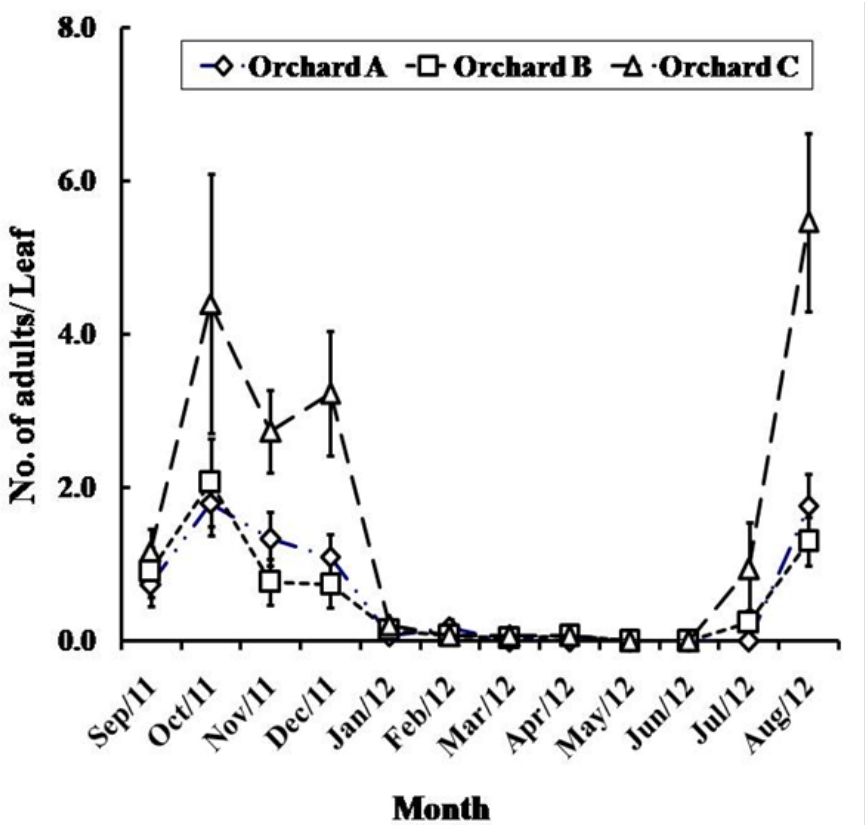

Fig. 4. Seasonal abundance of adults of *A. dispersus* on guava leaves in Bangladesh during September 2011 and August 2012.

Our result suggests that the maximum number of egg masses, nymphs, pupae and adults were found in the month of August to January. But no infestation was found in the month of February to July, probably the reasons that the spiraling whitefly could not survive due to high rainfall. This results also showed that the nymphal population were the highest followed by the pupae and adult. Temperature, rainfall, humidity fog and sunshine may have affect on whitefly population in guava orchards. The present result is matching with those of Horowitz (1986) who reported that a significant drop of whitefly populations at heavy rainy condition. Echelkraut and Cardona (1989) observed that dry conditions are more favorable for *Bemisia tabaci* than those of high precipitation. Salinas and Sumald (1994) reported the presence of whitefly year-round and showed the negative effect of high temperature and rainfall on the population densities of whitefly. Rashid et al. (2003) also stated the same reason for the decreased number of whitefly populations. The extreme relative humidity, either high or low was unfavorable for the survival of immature stages (Gerling et al. 1986, Gerling 1990). The seasonal distribution of spiraling whitefly highest in the month of August and followed by September to January and then decreased gradually while absent in April in ornamental plants (Alim et al. 2014).

Seasonal abundance of natural enemies

Two natural enemies *viz*. one predatory ladybeetle, *Scymnus* sp. and pupal parasitoid, *Encarsia* sp. were found in association with *A. dispersus* during the survey period in three guava orchards (Fig. 5). Results indicated that the highest number of ladybird beetle (0.20%) reached in the month of October in orchard B. On the other hand, the highest number of *Encarsia* sp. (0.15%) in the month of January in orchard B. So far no differences were observed among the recorded three orchards under study.

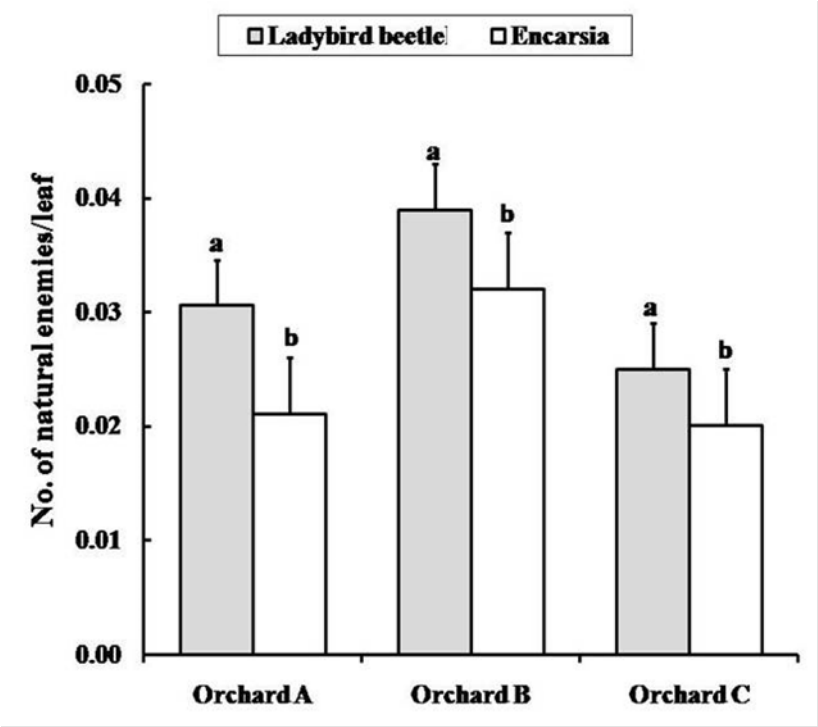

Fig. 5. Seasonal abundance of natural enemies of *A. dispersus* on guava orchards in Bangladesh during September 2011 and August 2012.

Variation in the number of natural enemies was statistically significant in different guava orchards. Mani and Krishnamoorrthy (1999) also found the same species of coccinellid predator and *Encarsia* sp. on whitefly in India. Rashid et al. (2003) cited that the release of coccinellid predator reduces *A. dispersus* population density. The population of ladybird beetle, *Scymnus* sp. and pupal parasitoid, *Encarsia* sp. were found during the survey period in ornamental plants (Alim et al. 2014). The experimental results showed that the presence of ladybird beetle was affected by the presence of associated ants. The association between ants and the whiteflies was found to be a very common phenomenon in guava orchards. It was also noticed that the presence of ladybird beetles and pupal parasites in the orchard only in the winter months when the whitefly population was comparatively higher. The unavailability of the predator in summer which was hot and humid indicated that the predators might have a temperature or prey density dependent relationship.

Seasonal abundance of sooty mould infested leaves

Sooty mould infestation per 30 leaves was highest in the month of October through January in all the studied orchards (Fig. 6). No sooty mould infestation was found from February to July. The month of November showed significantly highest sooty mould infestation ($F = 15.27$, $df = 11$, $P<0.001$).

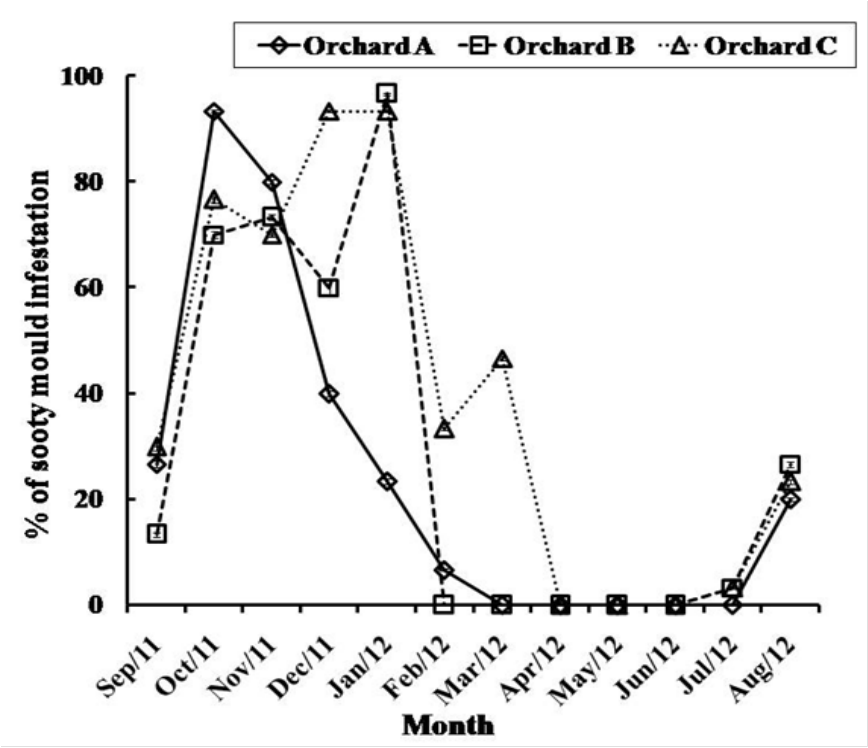

Fig. 6. Sooty mould infestation (%) on guava leaves in Bangladesh during September 2011 and August 2012.

Abundance of sooty mould infested leaves was statistically significant in different orchards. Our orchards results showed that abundance of sooty mould infested leaves was found only in the winter months when the whitefly population was comparatively higher. But no infestation was found in the months from April to July because of the high temperature, rainfall and humidity. Rashid et al. (2003) also mentioned similar reasons for reduced number of shooty mould infestation in guava orchards. Alim et al. (2014) stated that the highest number of sooty mould infected leaves was found in the month of October in ornamental plants.

Conclusion

The survey results indicated that the incidence of *A. dispersus* population was peak in the months of August to January while no infestation was found in the months from February to July because of high temperature and high rainfall. Thus the information of seasonal incidence of spiraling whitefly and its associated natural enemies might successfully contribute to the existing pest control methods and the development of new control strategies for guava orchards in Bangladesh.

Acknowledgement

The authors acknowledge the Institute of Training and Research (IRT), Hajee Mohammad Danesh Science and Technology University, Bangladesh for financial support.

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J. Bio-Sci. 24: 75-81, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

ROBUST LINEAR REGRESSION BASED SIMPLE INTERVAL MAPPING FOR QTL ANALYSIS WITH BACKCROSS POPULATION

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Abstract

Simple interval mapping (SIM) is one of the most important techniques for the identification of quantitative trait locus (QTL). Most of the approaches of SIM are very sensitive to phenotypic outliers and produce misleading results. There is a robust approach of SIM only for F_2 population. However, there is no robust SIM method for Backcross population. The objective was to develop a new approach of SIM with Backcross population which is robust against phenotypic outliers and performs almost the same as existing classical methods in absence of outliers. Maximum likelihood (ML) and linear regression (LR) based approaches of SIM are not robust against phenotypic outliers. In this research, we have developed a robust regression based SIM approach by maximizing β-likelihood function for Backcross population. The proposed method reduces to the LR-based SIM method when $\beta = 0$. To measure the performance of the proposed method in comparison of ML and LR based SIM with backcross population; we have generated phenotypic and genotypic data for Backcross population using simulation technique. LOD score profile plot shows that the highest peaks of LOD scores occur in the true QTL positions of the true chromosomes at true markers by all three methods for the uncontaminated dataset. However, in presence of outliers, only the proposed method gives the highest LOD score peaks at the true QTL positions on the true chromosomes. The simulation results showed that the proposed method improves performance over the existing SIM methods in presence of phenotypic contaminations.

Key words: Backcross population, beta-LRT criterion, maximum beta-likelihood estimation, QTL analysis, robustness, robust linear regression

Introduction

The rapid advancement in molecular biology has increased the availability of fine scale genetic markers which facilitate the wide use of QTL analysis in the genetic study of quantitative traits in bioinformatics. Liu (1997) and Wu et al. (2007) discussed various techniques of QTL mapping in their texts. Thoday (1961) first proposed the idea of using two markers to bracket a region for testing QTLs. Soller et al. (1976) examined the power of experiments at detecting linkage between a quantitative locus and a marker locus. Similar to Thoday's (1961), but much improved, method called interval mapping (IM) approach was proposed by Lander and Botstein (1989) which is based on linkage relationships between a QTL and flanking markers. Maximum likelihood (ML) based IM (Lander and Botstein 1989) and regression based IM (Haley and Knott 1992) are two most popular and widely used interval mapping approaches.

In practice, QTL effects are treated as either fixed or random (Xu 1998). In fixed effects QTL model, allelic substitution effects are usually estimated and tested, and QTL variance is calculated from estimated allelic

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effects. In random effects QTL model, the QTL effects and QTL variance are directly estimated and tested. Since the conditional expectations of the QTL genotype given the flanking marker genotype are unknown in MLE based IM model (Lander and Botstein 1989), this QTL effect model can be treated as a random effects model (REM). On the other hands, in the HK regression based IM model the conditional expectation of the QTL genotype given the flanking marker genotype is considered as fixed (Kao 2000) and this model can be treated as a fixed effect model (FEM).

The existing interval mapping based on REM (Lander and Botstein 1989) and FEM (Haley and Knott 1992) are two most popular and widely used methods for QTL analysis. But these methods are not robust against phenotypic contaminations. There is a regression based robust approach of SIM for QTL mapping only with F_2 population (Alam et al. 2015). In this work, we propose a robust method with FEM to perform QTL analysis for Backcross population. Also we have investigated the performance of the proposed method with the existing random effect QTL model and fixed effect QTL model for Backcross population by simulation study.

Materials and Methods

Linear regression based SIM approach for QTL detection with backcross population

Let us consider no epistatis between two QTLs, no interference in crossing over, and only one QTL in the testing interval. The fixed effect model for Backcross population, for testing a QTL within a marker interval, is define as

$$y_j = \mu + ax_{j|i} + u_j,\ i = 1, 2 \text{ and } j = 1, 2, \ldots, n \qquad (1)$$

where y_j is the phenotypic value of the j-th individual, μ is the general mean effect, $x_{j|i} = p_{j|1}$, a is the QTL additive effect and $u_j \sim NID(0,\sigma^2)$ is a random error. Here, $x_{j|i}$ is the conditional probability for QTL genotypes given the flanking marker genotypes. Since conditional expectation is equivalent to conditional probabilities of QTL genotypes (Kao 2000), $x_{j|i}$ is fixed for QTL genotypes given flanking marker genotypes. Since $x_{j|i}$ is fixed, so this model is called fixed effect model.

The conditional probabilities for QTL genotypes *QQ* and *Qq* given the flanking marker genotypes are denoted by $p_{j|1}$ and $p_{j|2}$, respectively. The conditional probabilities $p_{j|1}$ and $p_{j|2}$ are shown in Table 1 for Backcross population. In Table 1, p is defined as $p = r_{MQ}/r_{MN}$ where r_{MQ} is the recombination fraction between the left marker *M* and the putative QTL and r_{MN} is the recombination fraction between two flanking markers *M* and *N*. The possibility of a double recombination event in the interval is ignored.

Table 1. Conditional Probabilities of a putative QTL genotype given the flanking marker genotypes for a backcross population.

Marker genotypes	Expected frequency	QTL genotypes	
		QQ ($p_{j\|1}$)	*Qq* ($p_{j\|2}$)
MN/MN	$(1-r)/2$	1	0
MN/Mn	$r/2$	$(1-p)$	p
MN/mN	$r/2$	p	$(1-p)$
MN/mn	$(1-r)/2$	0	1

To investigate the existence of a QTL at a given position within a marker interval, we want to test the hypothesis H_0: $a = 0$ (i.e., there is no QTL at a given position) versus H_1: H_0 is not true. Under the normality assumption of error, the probability density function of the trait value (y) within each QTL genotype class is $N(\mu + ax_{jli}, \sigma^2)$.

Then the likelihood function for the parameters $\boldsymbol{\theta} = (\mu, a, \sigma^2)$ can be written as follows

$$L(\boldsymbol{\theta} \mid Y) = \prod_{i=1}^{n} \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{y_j - \mu - ax_{jli}}{\sigma} \right)^2 \right] \tag{2}$$

To test H_0 against H_1, the likelihood ratio test (LRT) statistic is defined as

$$LRT = -2\ln\left[\frac{\sup_{\Theta_0} L(\boldsymbol{\theta} \mid Y)}{\sup_{\Theta} L(\boldsymbol{\theta} \mid Y)} \right] = 4.608295 * \mathrm{LOD} \tag{3}$$

where, Θ_0 and Θ are the restricted (H_0) and unrestricted (H_1) parameter spaces.

The threshold value to reject the null hypothesis cannot be simply chosen from a chi-square distribution because of the violation of regularity conditions of asymptotic theory under H_0. The number and size of intervals should be considered in determining the threshold value. Since multiple tests are performed in mapping, the hypotheses are usually tested at every position of an interval and for all intervals of the genome to produce a continuous LRT statistic profile. At every position, the position parameter p is predetermined and only μ, a and σ^2 are involved in estimation and testing. If the tests are significant in a chromosomal region, the position with the largest LRT statistic is inferred as the estimate of the QTL position and the maximum likelihood estimates (MLEs) at this position are the estimates of μ, a and σ^2 obtained by iterative way.

The MLEs of the parameters μ, a and σ^2 are as follows

$$\hat{\mu} = \bar{y} - a\bar{x}, \hat{a} = \frac{\sum_{j=1}^{n} (x_{jli} - \bar{x})(y_j - \bar{y})}{\sum_{j=1}^{n} (x_{jli} - \bar{x})^2} \text{ and } \hat{\sigma}^2 = \frac{1}{n}\sum_{j=1}^{n} (y_j - \hat{\mu} - \hat{a}x_{jli})^2 \tag{4}$$

where $\bar{y} = \frac{1}{n}\sum_{j=1}^{n} y_j$ and $\bar{x} = \frac{1}{n}\sum_{j=1}^{n} x_{jli}, i = 1, 2.$

Obviously these ML estimates of μ, a and σ^2 are very much sensitive to outliers. Therefore, regression analysis by MLE produces misleading results in presence of contaminated data.

Robust linear regression based SIM for QTL detection with backcross population

The β-likelihood function (for details about β-likelihood (Mollah et al. 2007)) for $\boldsymbol{\theta}$ is given by

$$L_\beta(\boldsymbol{\theta}\,|\,Y)=\frac{1}{\beta}\left[\frac{1}{nl_\beta(\boldsymbol{\theta})}\sum_{t=1}^{n} f_\theta^{\beta}(y_t)-1\right] \tag{5}$$

The β-likelihood equation is obtained as

$$\sum_{j=1}^{n}(y_j-\mu-ax_{j|i})w(y_j\,|\,\boldsymbol{\theta},x_{j|i})x_{kj}=0;\,k=0,1,2 \tag{6}$$

where $x_{0j}=1$ for all $j=1, 2, \ldots, n$ and $w(y_j\,|\,\boldsymbol{\theta},x_{j|i})=\exp\left[-\frac{\beta}{2\sigma^2}(y_j-\mu-ax_{j|i})^2\right]$ for $i=1, 2$. The function $w(y_j|\boldsymbol{\theta}, x_{j|i})$ is the weight function which produces almost zero weight for the outlying observations. Solving equation (6), we get the proposed estimates of the parameters $\boldsymbol{\theta}$ as

$$\hat{\mu}=\bar{y}_w-a\bar{x}_w,\ \hat{a}=\frac{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})(x_{j|i}-\bar{x}_w)(y_j-\bar{y}_w)}{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})(x_{j|i}-\bar{x}_w)^2}\ \text{and}$$

$$\hat{\sigma}^2=\frac{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})(y_j-\hat{\mu}-\hat{a}x_{j|i})^2}{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})} \tag{7}$$

where $\bar{y}_w=\frac{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})y_j}{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})}$ and $\bar{x}_w=\frac{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})x_{j|i}}{\sum_{j=1}^{n}w(y_j\,|\,\boldsymbol{\theta},x_{j|i})},\ i=1,2.$

To test H_0: $a=0$ against H_1: H_0 is not true, the proposed test criterion is defined as

$$\lambda_\beta=2n[L_\beta(\hat{\boldsymbol{\theta}}_1\,|\,Y)-L_\beta(\hat{\boldsymbol{\theta}}_0\,|\,Y)],\ \text{where}\ \hat{\boldsymbol{\theta}}_0=(\hat{\mu},\hat{\sigma}^2)\ \text{and}\ \hat{\boldsymbol{\theta}}_1=(\hat{\mu},\hat{a},\hat{\sigma}^2). \tag{8}$$

By permutation test, we compute the *p*-value for testing H_0 vs H_1 using the following formula

$$p=\sum_{k=1}^{N_p}I_{\left[\hat{\lambda}_{\beta(k)}\le\hat{\lambda}_\beta\right]}/N_p \tag{9}$$

where N_p is the number of permutation under H_0 and $\hat{\lambda}_\beta$ is the estimate of λ_β for the original dataset and $\hat{\lambda}_\beta(k)$ is the estimate of λ_β for the k-th permutation of the values of the response variable. Note that, for $\beta\to 0$, $\hat{\lambda}_\beta$ reduces to the approximate χ^2 distribution.

Simulated data

To measure the performance of the proposed method in comparison of the fixed effect and random effect models for QTL mapping with Backcross population, we have generated phenotypic and genotypic data for Backcross population using simulation technique. We have considered two unlinked QTLs, total 10 chromosomes and 11 equally spaced markers in each of the 10 chromosomes, where any two successive marker interval size is 5 cM. The true QTL position is located in chromosome 2, 3and 5 at marker 5 (locus position 20 cM). The true values for the parameters in the fixed effect model are assumed as $\mu = 0.5$, $a = 0.8$, $d = 0.4$ and $\sigma^2 = 0.5$. We have generated 250 trait values with heritability $h^2 = 0.20$ which means that 20% of the trait variation is controlled by QTL and the remaining 80% is subject to the environmental effects (random error). To investigate the robustness of the proposed method in a comparison of the REM and FEM methods, we contaminated 12% trait values in this dataset by outliers. To perform the simulation study we have used R/qtl software (Broman et al. 2003, homepage: http://www.rqtl.org/).

Results and Discussion

Table 2 shows QTL positions (i.e., chromosome, marker and locus position) identified by REM, FEM and the proposed method. Fig. 1a and 1b are representing the scatter plots of 250 trait values in presence and absence of outliers, respectively. Then we computed LOD scores based on REM, FEM and the proposed methods for both types of data sets. Fig. 1c and 1d are showing the LOD scores profile plots for the uncontaminated and contaminated datasets, respectively. In the LOD scores profile plots the dotted, two dash and solid lines represent the LOD scores at every 1cM position in the chromosomes for REM, FEM and the proposed method with $\beta = 0.2$, respectively.

It is seen that the highest LOD score peak occurs in the true QTL position of the true chromosome 2, 3 and 5 at marker 5 (locus position 20 cM) by all three methods for the uncontaminated dataset. However, in presence of outliers, the highest LOD score peak occurs in the true QTL position by the proposed method only (Fig. 1d).

Table 2. QTL positions identified by each method in absence and presence of outliers.

Methods	True QTL positions	Identified QTL positions	
		In absence of outliers	In presence of outliers
REM	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	REM fails identify any QTL on any chromosome.
FEM	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	(i) On chromosome 1 at marker 10 (locus position 45 cM). (ii) On chromosome 2 at marker 5 (locus position 20 cM). (iii) On chromosome 5 at marker 1 (locus position 0 cM). (iv) On chromosome 10 at marker 11 (locus position 50 cM)
Proposed model	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.	On chromosomes 2, 3 and 5 at marker 5 (locus position 20 cM) for each chromosome.

From Table 2 and Fig. 1 we observe that all of the 3 methods (REM, FEM and proposed method) identify the true QTL positions correctly in absence of outliers. But in presence of outliers the REM fails to identify any significant QTL position and the FEM identify QTLs on chromosomes 1 at marker 10 (locus position 45 cM), on chromosome 2 at marker 5 (locus position 20 cM), on chromosome 5 at marker 1 (locus position 0 cM) and on chromosome 10 at marker 11 (locus position 50 cM). The positions on chromosome 5 at marker 1 and on chromosome 10 at marker 11, identified by FEM, are not the true position of QTLs. However, in presence of outliers, the proposed method identify the QTLs on chromosome 2, 3 and 5 at marker 5 (locus position 20 cM) which are the true QTL positions.

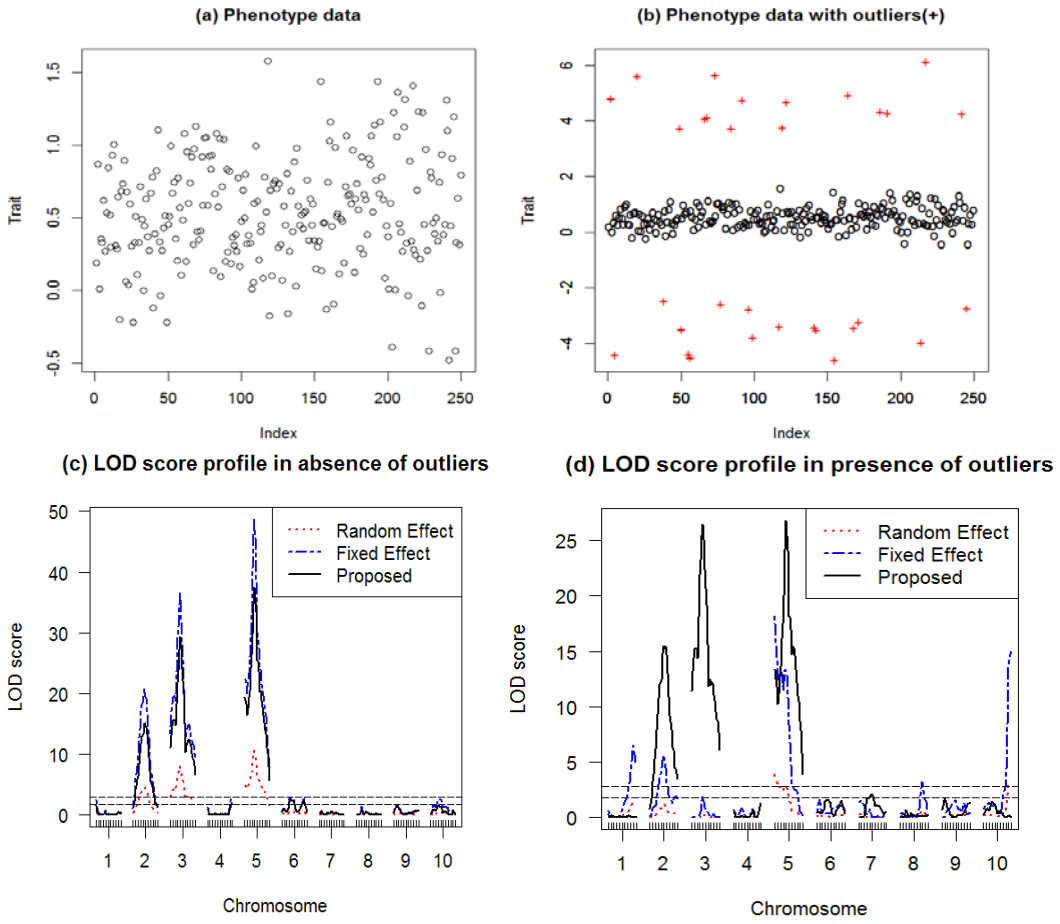

Fig. 1. Simulated phenotypic observations in (a) absence and (b) presence of 12% outliers, and LOD score profile in (c) absence and (d) in presence of 12% outliers.

Hence, in presence of outliers, the classical methods of SIM (REM and FEM) fail to identify the all the true QTL positions whereas the proposed method successfully identifies all the true QTL positions. Also in absence of outliers the proposed method is working as the classical methods.

Conclusion

Under this study we have proposed a new robust regression based simple interval mapping approach for QTL analysis by maximum β-likelihood estimation with Backcross population. The performance of the proposed method is controlled by the tuning parameter β. An appropriate value for the tuning parameter β can be selected by cross validation. The proposed method reduces to the traditional interval mapping approach when the tuning parameter $\beta = 0$. Simulation results show that the proposed method significantly improves the performance over the classical simple interval mapping approaches in presence of phenotypic outliers. Also in absence of outliers it shows similar performance to the classical methods of SIM.

Acknowledgement

We would like to acknowledge to the HEQEP Sub-Project (CP-3603, W2, R3) for providing financial support for this study.

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J. Bio-Sci. 24: 83-85, 2016 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

-Short communication

EFFECT OF DUAL INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGUS AND *RHIZOBIUM* ON CHLOROPHYLL CONTENT OF PIGEON PEA [*CAJANUS CAJAN* (L.) MILL SP.]

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Soil microorganisms are known to regulate mycorrhizal formation and function conversely. Mycorrhiza affects the establishment of rhizosphere population. Some interaction between mycorrhizae and soil microorganisms involve nutrient recycling. Hence, they have an impact on plant and nutrient (Kapoor and Mukerji 1998). The symbiotic relation between arbuscular mycorrhizal (AM) fungi and the host plants has been studied traditionally in terms of benefits to the individual plant and fungi (Smith and Smith 1996, Lakshman 1999). AM fungal association can affect the host plants in terms of stomata movement to increase chlorophyll content and the rate of transpiration and photosynthesis (Panwar 1991, Bheemareddy and Lakshman 2011). Mycorrhizal colonization is of particular value to legumes because it can increase the phosphorus uptake: nodulation and symbiotic nitrogen fixation by rhizobial require adequate supply of phosphorus, and restricted root system leads to poor competition for soil phosphorus (Carling et al. 1978, Bagyaraj 2006, Lakshman 2009).

Pigeon pea (*Cajanus cajan* L.) is probably a native of tropical Africa, and was introduced perhaps 3,000 years ago into India. It is chiefly grown in Madhya Pradesh, Bihar, Andra Pradesh, Maharashtra, Uttar Pradesh, and Karnataka. Both the immature and ripen fruits are used for human food as a good source of protein. The leaves and twigs are used as fodder. The pericarp and husk, separated in threshing are used as cattle feed. The enzyme urease, obtained from it, is required for estimation of urea in blood, urine etc. Livestock and poultry are very much fond of it. It is chiefly consumed in South Indian homes. In fact, research on chlorophyll content of this plant with inoculation of AM fungus (*Glomus macrocarpum*) and *Rhizobium leguminosorum* is very meager, therefore the present study was undertaken. Seeds of Pigeon pea *Cajanus cajan* Mill sp. were obtained from the seed bank unit of University of Agricultural Science Dharwad – 580005, in Karnataka state. Only healthy seeds were selected for study. Seedlings were raised in earthen pots measuring 20 × 25 cm (length × breadth) diameter containing 4 kg sterile mixture sand and soil in equal proportion. Before sowing the seeds of Pigeon pea were inoculated with AM fungus *G. macrocarpum* (15 g) dry mixed inoculum was placed as a thin layer, just below 4cm soil surface of experimental pots. One seedling was left per pot and without inoculation as control. Leaves were collected for analyses of chlorophyll -A and chlorophyll -B after 30, 60, 90 days of inoculation. The chlorophyll content was estimated following the procedure of Arnan (1949). All the experiments were carried out in triplicate under green house condition.

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Table 1. Showing the effect of AM fungus and *Rhizobium* on chlorophyll content of pigeon pea at different intervals of time (30, 60 and 90 days) in unsterile soil.

Treatment	Chlorophyll A (mg/g)/ Day			Chlorophyll B (mg)/ Day			Total Chlorophyll (mg)/ Day		
	30	60	90	30	60	90	30	60	90
Control	0.631	0.723	0.871	0.584	0.689	0.717	1.215	1.412	1.588
Glomus macrocarpum	0.891	0.930	1.124	0.721	0.749	0.803	1.612	1.679	1.927
Rhizobium leguminosorum	0.862	0.894	1.015	0.645	0.734	0.811	1.507	1.628	1.826
G. ma + Rh	0.941	1.842	2.019	0.672	0.739	0.823	2.783	2.581	2.842

Table 2. Showing the effect of AM fungus and *Rhizobium* on chlorophyll content of pigeon pea at different intervals of time (30, 60 and 90 days) in sterile soil.

Treatment	Chlorophyll A (mg)/ Day			Chlorophyll B (mg) Day			Total Chlorophyll (mg/g)/ Day		
	30	60	90	30	60	90	30	60	90
Control	0.631	0.721	0.783	0.489	0.593	0.616	1.12	1.31	1.40
Glomus macrocarpum	0.841	0.924	1.116	0.521	0.633	0.782	1.37	1.55	1.90
Rhizobium leguminosorum	0.852	0.896	1.014	0.508	0.612	0.774	1.36	1.51	1.79
G. ma + Rh	0.937	1.841	2.115	0.678	0.738	0.823	1.61	2.52	2.94

G. ma- *Glomus macrocarpum*, Rh- *Rhizobium*

The analysis of chlorophyll A, B, and total chlorophyll content of leaf revealed a significant variation, due to Pigeon pea plants inoculated with AM fungus (*Glomus macrocarpum*) alone or in combination with *Rhizobium*. The total chlorophyll content was highest in dual inoculated plants grown in sterilized soil than that of plants grown in unsterile soil (Table 1 and 2). Such and increase might be due to transpiration or increased growth (Hayman 1983,Sampathkumar and Ganeshkumar 2003) or due to the presence of a large number of chlorophyll in the bundle sheath of inoculated leaves (Krishna and Bagyaraj 1984, Rajashekharan and Nagarajan 2005). Our study is par with earlier studies of other workers (Bhavani et al. 1998, Katiyar et al. 1998, Baqual et al. 2005, Rajashekharan and Nagarajan 2005), that chlorophyll content is higher in the leaves of bio inoculants inoculated plants compare to noninoculated (Control) plants, as biochemical characters like phenols, proteins and chlorophylls may play a vital role in making plants resistant to

pathogens. Similar reports are also available that the AM fungi association with the N- fixing bacteria can increase the N-fixing capacity of many crop plants, mainly legumes (Patterson et al. 1990).

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CONTENTS