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Full Length Research Paper

In vitro selective cytotoxicity of activated parasporal proteins produced by *Bacillus thuringiensis* serovars *kumamotoensis* and *tohokuensis* against human cancer cell lines

Maher Obeidat

Department of Biotechnology, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt 19117, Jordan.

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The anti-cancer activity of alkali-solubilized protease-activated parasporal proteins produced by 78 local *Bacillus thuringiensis* strains and 14 reference *B. thuringiensis* strains was screened against five human cancer cell lines (CACO-2, Hep2, HepG2, K562, and MCF-7). Activated parasporal proteins were tested for their hemolytic activity against human erythrocytes. It was found that activated parasporal proteins of 25 local *B. thuringiensis* strains and 9 reference strains were non-hemolytic. Non-hemolytic parasporal proteins produced by 9 local *B. thuringiensis* strains were found to exhibit no to low cytotoxicity against human non-cancerous Hs27 cells. Out of them, activated parasporal proteins of two local *B. thuringiensis* strains (J61; *B. thuringiensis* serovar *kumamotoensis* and J72; *B. thuringiensis* serovar *tohokuensis*) were found to produce high to very high *in vitro* selective cytotoxicities, preferentially toxic to cancerous cells, against all cancer cell lines used in this study. This is the first observation of the anti-cancer activity from *B. thuringiensis* serovar *kumamotoensis*. Based on IC_{50} values, activated parasporal proteins of J61 strain produced the most significant cytotoxicity against all cancer cell lines. Furthermore, CACO-2 and MCF-7 cells were found to be the most sensitive. Thus, parasporal proteins produced by *B. thuringiensis* serovar *kumamotoensis* strain J61 and/or *B. thuringiensis* serovar *tohokuensis* strain J72 may be used as alternative or improving means for current cancer therapy.

Key words: *Bacillus thuringiensis*, *kumamotoensis*, *tohokuensis*, parasporal, cancer.

INTRODUCTION

Cancer is a major public health problem worldwide and is the second cause of death after cardiovascular diseases (coronary heart disease and hypertension) in Jordan as well as in the world. According to the Ministry of Health annual report (MOH, 2013), cancer of all types (in particular breast cancer, colorectal cancers, lymphomas,

E-mail: obeidat@bau.edu.jo. Tel: 00966775609846.

lung cancer, and leukemia) was responsible for the death of over 10,000 Jordanians from 1996 until now. A total of 8,744 new cancer cases were registered by Jordan Cancer Registry (JCR) in 2013. Out of new cases of cancer recorded amongst Jordanians in 2013, colorectal cancer (12.7%) followed by lung cancer (11%) were the most common among Jordanian males. Whereas, breast cancer (36.5%) followed by colorectal cancer (9.4%) were the most common among Jordanian females. The spread of cancer is increasing over the world especially in developing countries (Plummer et al., 2016). The number of new cancer cases diagnosed among Jordanians has increased up to 51% in the past ten years with 0.9:1 male to female ratio according to JCR.

There is a great deal of controversy within the medical community over what kind of medical treatment is most efficacious in treating cancer. Most conventional medical treatments for cancer are of little help, so alternative techniques are required to be developed to target cancer.

In this research, the possibility of using *Bacillus thuringiensis* (Bt) as a source of anti-cancer compounds that are usually too difficult to create synthetically will be investigated. The bacterium *B. thuringiensis* is a Gram-positive spore-forming soil bacterium. Several strains of this bacterium produce crystalline parasporal proteins during sporulation. These parasporal proteins often contain δ-endotoxin proteins (known as insecticidal Cry proteins) that can exhibit strong insecticidal activity against agriculturally and medically important insect pests (Saraswathy and Kumar, 2004). It has long been believed that *B. thuringiensis* is characterized by insecticidal activity associated with its parasporal proteins. However, several studies (Ohba et al*.,* 1988; Ohba, 1996; Roh et al*.,* 1996; Mizuki et al*.,* 1999a) have demonstrated that *B. thuringiensis* strains producing non-insecticidal parasporal proteins are more widely distributed than insecticidal ones. In the beginning of this century, many efforts were oriented toward studying the biological activities of non-insecticidal parasporal proteins yet to be discovered. Mizuki et al*.* (1999b) reported cytotoxicity against human leukemic T cells and other human cancer cells for parasporal proteins produced by some non-insecticidal *B. thuringiensis* strains. Moreover, it was found that these non-insecticidal Cry proteins, designated parasporins, are capable of preferentially killing cancer cells upon proteolytic degradation (Mizuki et al*.,* 1999b, 2000; Yamashita et al*.,* 2000; Ito et al*.*, 2004; Okumura et al*.*, 2004; Katayama et al*.,* 2005; Hayakawa et al*.*, 2007; Jung et al*.*, 2007; Uemori et al*.*, 2008; Yasutake et al*.*, 2008; Nagamatsu et al*.*, 2010). Six parasporin families, including 19 parasporin toxins isolated from *B. thuringiensis* strains, were identified by the Committee of Parasporin Classification and Nomenclature (Okumura et al., 2010; Wong, 2010). For example, parasporin-1 was isolated from *B. thuringiensis* strain A1190 (Mizuki et al*.,* 1999b, 2000). This parasporin when degraded by proteases exhibited selective cytotoxicity against cancer cells such as human leukemic T cells (Mizuki et al*.,* 2000). It was found that parasporin-1 activates apoptotic pathway (Katayama et al*.,* 2007). Activated parasporin-2 isolated from *B. thuringiensis* A1547 belonging to serovar *dakota* showed extremely high cytotoxicity against different kinds of cancer cells including leukemic T cells such as MOLT-4, Jurkat, and HL-60 (Kim et al*.,* 2000; Ito et al*.,* 2004; Kitada et al*.,* 2006). It was found that the cytotoxic effect of parasporin-2 is non-apoptotic toward cancer cells but causes swelling of the susceptible cells (Ito et al., 2004). Moreover, another parasporin, called parasporin-3, isolated from *B. thuringiensis* isolate A1462 exhibited limited cytotoxicity against human leukemic T cells (Yamashita et al., 2000, 2005). It was hypothesized that parasporin-3 kills cancer cells by a receptor-mediated mechanism (Yamashita et al., 2005). An important parasporin known as parasporin-4 was obtained from *B. thuringiensis* strain A1470 which belongs to serovar *shandongiensis* induced necrosis-like cytotoxicity against human leukemic T cells (Lee et al*.,* 2000; Okumura et al*.,* 2005; Saitoh et al*.,* 2006). A cytotoxic protein toward the leukemic T cell, MOLT-4, was also isolated from *B. thuringiensis* serovar *coreanensis* strain A1519 (Namba et al., 2003). Parasporin-5, purified from *B. thuringiensis* serovar *tohokuensis* strain A1100, was found to exhibit cytocidal activity against human leukemic T cells (Ekino et al., 2014). A novel parasporin isolated from *B. thuringiensis* strain M019 was classified into a new parasporin family (parasporin-6) preferentially kill human hepatocyte cancer HepG2 cells and uterus cervix cancer HeLa cells (Nagamatsu et al*.*, 2010).

Efforts dedicated to screening microbial natural products for cancer therapy are very limited. Therefore, the current research is an important advance in the fight against cancer because bacterial metabolites are not well explored for their effectiveness in cancer therapy. These metabolic products may become an alternative or a supplement to current cancer therapies. Thus, this research aimed to test *B. thuringiensis* parasporal proteins against several cancer cells as alternative or improving means for current cancer therapy.

MATERIALS AND METHODS

B. thuringiensis strains and growth conditions

Seventy-eight local Jordanian *B. thuringiensis* strains representing 14 serovars (*aizawai, entomocidus, higo, israelensis, jordanica, kenyae, kumamotoensis, kurstaki, malaysiensis, morrisoni, pakistani, sooncheon, thuringiensis,* and *tohokuensis*), four nonserotypable (NSP) strains (unable to react with antisera), and one autoagglutinated (AA) strain were used in this study. These strains were previously isolated from different Jordanian habitats and classified by serotyping (Obeidat et al*.,* 2000; Khyami-Horani, 2003; Al-Momani et al., 2004). Fourteen reference strains obtained from International Entomopathogenic *Bacillus* Collection Center (IEBC), Institute Pasteur, Paris, including, *B. t*. *thuringiensis* T01001, *B. t. thuringiensis* T01022, *B. t. kurstaki* T03A001, *B. t.*

kurstaki HD1 T03A005, *B. t. kurstaki* T03A361, *B. t. kenyae* T04B001, *B. t. entomocidus* T06001, *B. t. aizawai* T07001, *B. t. morrisoni* T08001, *B. t. tolworthi* T09001, *B. t. darmstadiensis* T10001, *B. t. pakistani* T13001, *B. t. israelensis* T14001, and *B*. t. *israelensis* 1884 T14007 were also used. Among the *B. thuringiensis* strains, 35 strains showed no insecticidal activities against diptera and lepidoptera (Saadoun et al., 2001; Khyami-Horani, 2002; Obeidat et al*.,* 2004, 2012).

Cultures were grown overnight at 37°C on T_3 medium (0.3% tryptone, 0.2% tryptose, 0.15% yeast extract, 0.05 M sodium phosphate, and 0.005% $MnCl_2$) (Travers et al., 1987) and maintained as suspensions of spores and cells at -70°C with 20% (v/v) glycerol.

Solubilization of *B. thuringiensis* parasporal proteins

Bacterial strains were cultured in 75 ml of T_3 broth (Travers et al., 1987). These cultures were incubated at 150 rpm for 3 days at 37°C, and then left at 4°C for 1 day. Sporulated cultures of *B. thuringiensis* were centrifuged at 5000 rpm for 10 min at 4°C, and washed three times with distilled water before solubilization (Saitoh et al*.,* 1996). The crude parasporal proteins were solubilized in 5 ml of 50 mM Na_2CO_3 (pH 10) containing 10 mM dithiothreitol (DTT) and 1 mM EDTA for 1 h at 37°C (Mizuki et al*.,* 1999b). After solubilization, the mixture was centrifuged at 14000 rpm for 5 min at 4°C, and the pH of the supernatant was adjusted to 8 by 1 M HCl then passed through a 0.45-μm membrane filter.

Activation of *B. thuringiensis* parasporal proteins

Solubilized parasporal proteins were activated by proteolysis in 0.3 mg/ml proteinase-K at 37°C for 1.5 h. After that, 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to stop the proteolytic reaction (Mizuki et al*.,* 2000). The mixture was then filtered through a 0.45-μm membrane filter and the filtered fluid was stored at 4°C until use.

Protein quantification

The protein concentration in the filtered fluid was determined by the method of Bradford (1976). Bradford procedure was performed by using bovine serum albumin (BSA) as standard. After assessment of protein concentration by spectrophotometry at 595 nm absorbance, the protein concentration in each sample was adjusted to 1 mg /ml.

Hemolytic activity

Hemolytic activity was tested on blood agar medium using fresh human erythrocytes (5%). 50 µl of activated parasporal proteins was added into each well (5 mm i.d.) prepared on blood agar plates. The type of hemolysis was determined after incubation of plates at 37°C for 48 h (Carillo et al., 1996).

Cells and culture conditions

Six human cell lines were used in this study, including non-cancerous normal cells (Hs27; human foreskin fibroblast cell line) that was used as a non-transformed control and five cancerous cells (CACO-2; human colorectal adenocarcinoma cell line, Hep2; human larynx epidermoid carcinoma cell line, HepG2; human liver hepatocellular carcinoma cell line, K562; human leukemic T cells, and MCF-7; human breast cancer cell line) that were kindly supplied by Dr. Saeid Ismaeil (Faculty of Medicine, University of Jordan).

The non-adherent human leukemia cancer cell line K562 was grown in Roswell Park Memorial Institute Medium (RPMI 1640) medium at pH 7.4, supplemented with 10% fetal bovine serum (FBS), 40 µg/ml gentamicin, 50 µM 2-mercaptoethanol, 10 mM N-2-hydroxyethylpiperzine-N-2-ethane sulfonic acid (HEPES), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 incubator (Freshney, 2005). The K562 cells were harvested, subcultured, and reseeded in fresh medium at ~10^5 cells/ml every 48 h.

The adherent human cancer cell lines (CACO-2, Hep2, HepG2, and MCF-7) and the adherent non-cancerous Hs27 cell line were grown in Dulbecco's modified Eagle's medium (DMEM), pH 7.4, supplemented with 10% FBS, 40 µg/ml gentamicin, 50 µM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and they were harvested at ~70% confluence and subcultured every 48 h at 37°C in a humidified 5% CO_2 incubator (Freshney, 2005). To harvest the adherent cells, growth medium was removed and cells were washed with phosphate buffer saline (PBS). To produce a cellular suspension, a cell dissociation solution made of 1X trypsin-EDTA was added and incubated at 37°C for 5 min in a humidified 5% CO_2 incubator. Trypsinized cells were reseeded in fresh medium at ~10^5 cells/ml and incubated at 37°C in a humidified 5% CO_2 incubator.

Screening of anti-cancer activity

For screening assay, 20 µl of non-hemolytic proteinase K-activated parasporal proteins (1 mg/ml constant concentration, that is, 20 µg/well) was added to 200 µl of harvested Hs27 cells in fresh DMEM, mixed thoroughly by pipetting and 180 µl medium were loaded into each well of 96 well micro test plate to bring the total volume to 500 µl/well. Cells were plated at a density 4×10^4 cells/well, and counted by hemocytometer. Then, the 96 well micro test plates were incubated at 37°C in a humidified 5% CO_2 incubator for 48 h. At the end of incubation time, the viability of cells was assessed by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983; Heiss et al., 1997). In the assay, each well of the 96 well microtest plate received 40 µl (50 µg) of MTT and incubated at 37°C for 4 h in a humidified 5% CO_2 incubator. After that, 100 µl of dimethyl sulfoxide (DMSO) was added. The optical densities were measured at 450 nm with 630 nm reference wavelength using enzyme-linked immunosorbent assay (ELISA) microplate reader. Each treatment was performed in triplicate and repeated five times. The survival rate was determined by comparing the average of absorbance values with that in the control without parasporal proteins.

To determine the anticancer activities, the non-hemolytic parasporal proteins that exhibited no cytotoxicity against normal Hs27 cells were tested against cancer cell lines (CACO-2, Hep2, HepG2, K562, and MCF-7). The cytotoxicity assay was performed for adherent cell lines in the same manner as for Hs27 cells. Likewise, the cytotoxicity was tested on the non-adherent K562 cells in the same manner as for adherent cells but using RPMI 1640 medium instead of DMEM medium.

***In vitro* selective cytotoxicity**

The cytotoxicity of non-hemolytic protease-activated parasporal proteins that exhibited selective cytotoxicity (that is, non-toxic to non-cancerous Hs27 cell line but toxic to other cancerous cell lines) was performed in the same fashion as in the anticancer screening assay but using six concentrations (20, 10, 5, 1, 0.5, and 0.1 µg/ml)

Table 1. *In vitro* hemolytic activity of proteinase K-activated parasporal proteins produced by *B. thuringiensis* strains.

B. thuringiensis **serovar**	**Number of *B. thuringiensis* strains (number of reference *B. thuringiensis* strains)**	**Number of *B. thuringiensis* strains (number of reference *B. thuringiensis* strains)**		
		α-Hemolysis	**β-Hemolysis**	**γ-Hemolysis**
sooncheon	1 (0)	-	-	1 (0)
higo	1 (0)	-	-	1 (0)
kumamotoensis	2 (0)	1 (0)	-	1 (0)
tohokuensis	1 (0)	-	-	1 (0)
aizawai	1 (1)	1 (0)	-	0 (1)
entomocidus	1 (1)	-	-	1 (1)
jordanica	1 (0)	-	-	1 (0)
thuringiensis	2 (2)	0 (1)	-	2 (1)
malaysiensis	3 (0)	-	-	3 (0)
pakistani	5 (1)	3 (0)	1 (1)	1 (0)
morrisoni	2 (1)	1 (0)	-	1 (1)
kenyae	7 (1)	3 (0)	2 (0)	2 (1)
kurstaki	20 (3)	10 (1)	5 (1)	5 (1)
israelensis	26 (2)	20 (0)	2 (1)	4 (1)
tolworthi	0 (1)	-	-	0 (1)
darmstadiensis	0 (1)	-	-	0 (1)
Nonserotypable	4 (0)	2 (0)	1 (0)	1 (0)
Autoagglutinated	1 (0)	-	1 (0)	-
Total	78 (14)	41 (2)	12 (3)	25 (9)

of activated parasporal proteins. The mortality of cells was monitored after 48 h by MTT assay.

Statistical analysis

After treatment with MTT assay, the obtained absorbance values were corrected by subtracting the average absorbance of blank (contains the growth medium) from the average absorbance of vehicle (contains the growth medium and cells) and average absorbance of treatment (contains the growth medium, cells, and activated parasporal proteins). To calculate inhibition percentage of cells, the formula "Inhibition% = ((A-B)/A)×100" was used, where *A* is the vehicle absorbance and *B* is the treatment absorbance.

The median inhibitory concentration (IC_{50}) was determined by comparing the average mortality values of the six concentrations of activated parasporal proteins with that in the control without activated parasporal proteins. Each treatment was achieved in triplicate. The IC_{50} values, regression equations, and correlation coefficients (R^2) were determined by non-linear regression analysis (MS Excell, Microsoft Co., 2010). The 95% confidence limits (CLs) were also calculated using MS Excel (2010). The statistical outliers in data were excluded using Q-Test.

RESULTS

The current study was performed to screen the anti-cancer activity of proteinase K-activated parasporal proteins produced by 78 local *B. thuringiensis* strains and 14 reference *B. thuringiensis* strains against five human cancer cell lines, including, colorectal adenocarcinoma CACO-2 cells, larynx epidermoid carcinoma Hep2 cells, liver hepatocellular carcinoma HepG2 cells, leukemic K562 cells, and breast cancer MCF-7 cells.

As shown in Table 1, activated parasporal proteins of 25 *B. thuringiensis* strains and 9 reference strains were found to be non-hemolytic (γ-type). The non-hemolytic parasporal proteins of the local 25 *B. thuringiensis* strains belonged to 13 serovars (*sooncheon, higo, kumamotoensis, tohokuensis, entomocidus, jordanica, thuringiensis, malaysiensis, pakistani, morrisoni, kenyae, kurstaki,* and *israelensis*) and one NSP strain (Table 1). The non-hemolytic activated parasporal proteins of the reference strains were found to belong to nine *B. thuringiensis* serovars (*aizawai, entomocidus, thuringiensis, morrisoni, kenyae, kurstaki, israelensis, tolworthi, and darmstadiensis*) whereas, the remaining hemolytic *B. thuringiensis* strains were displayed either by α-hemolysis (41 *B. thuringiensis* strains and 2 reference strains) or β-hemolysis (12 *B. thuringiensis* strains and 3 reference strains). The AA strain exhibited α-hemolysis.

Proteinase K-activated parasporal proteins of the 34 *B. thuringiensis* strains (25 local strains and 9 reference strains) that exhibited no hemolysis against human erythrocytes were screened for their cytotoxicity against normal non-cancerous human Hs27 cells (Table 2). The colorimetric MTT assay was used to determine the degree of cytotoxicity of activated parasporal proteins against Hs27 cells. It was found that activated parasporal

Table 2. *In vitro* cytotoxicity of non-hemolytic proteinase K-activated parasporal proteins produced by *B. thuringiensis* strains against normal human Hs27 cells.

***B. thuringiensis* serovar**	**Cytotoxicity degree against Hs27[a]**					
	-	**±**	**+**	**++**	**+++**	**++++**
sooncheon	1	0	0	0	0	0
higo	0	0	0	0	1	0
kumamotoensis	1	0	0	0	0	0
tohokuensis	1	0	0	0	0	0
aizawai	0	0	0	1	0	0
entomocidus	0	0	0	1	0	1
jordanica	0	0	0	0	1	0
thuringiensis	1	0	0	1	0	1
malaysiensis	0	0	0	1	2	0
pakistani	0	0	0	0	1	0
morrisoni	0	1	0	0	1	0
kenyae	1	1	0	0	0	1
kurstaki	1	0	1	2	1	1
israelensis	0	0	0	0	1	4
tolworthi	0	0	0	0	0	1
darmstadiensis	0	0	0	0	0	1
Nonserotypable	0	0	0	0	1	0
Total	6	2	1	6	9	10

[a]The degree of cytotoxicity was graded on the basis of the relative value of absorbance to the vehicle: ++++, very high (<0.1); +++, high (0.1 to <0.4); ++, moderate (0.4 to <0.7); +, low (0.7 to <0.9); ±, very low (0.9 to <0.95); -, non-toxic (≥0.95).

Table 3. *In vitro* cytotoxicity of non-hemolytic proteinase K-activated parasporal proteins produced by *B. thuringiensis* strains against five cancer cell lines.

***B. thuringiensis* strain**	***B. thuringiensis* serovar**	**Cytotoxicity degree[a]**					
		Hs27	**CACO-2**	**MCF-7**	**K562**	**Hep2**	**HepG2**
J61	*kumamotoensis*	-	++++	+++	++++	++++	++++
J72	*tohokuensis*	-	++++	++++	+++	+++	++++

[a]The degree of cytotoxicity was graded as shown in Table 2.

proteins produced by nine local *B. thuringiensis* strains of seven serovars (*sooncheon, kumamotoensis, tohokuensis, thuringiensis, morrisoni, kenyae,* and *kurstaki*) exhibited no to low cytotoxicity against Hs27 cells (Table 2). Whereas, the non-hemolytic parasporal proteins of the remaining *B. thuringiensis* strains (16 local strains and 9 reference strains) were found to exhibit moderate to very high cytotoxicity against Hs27 cells.

The activated parasporal proteins of *B. thuringiensis* strains (9 strains) that exhibited no hemolysis against human erythrocytes and no (-) to low (+) cytotoxicities against normal non-cancerous human Hs27 cells were examined for their selective cytotoxicity against colorectal adenocarcinoma CACO-2 cells, larynx epidermoid carcinoma Hep2 cells, liver hepatocellular carcinoma HepG2 cells, leukemic K562 cells, and breast cancer MCF-7 cells (Table 3). It was found that activated parasporal proteins of two *B. thuringiensis* strains (J61; *B. thuringiensis* serovar *kumamotoensis* and J72; *B. thuringiensis* serovar *tohokuensis*) produced high to very high (degree of cytotoxicity is greater than 90%) selective cytotoxicities against cancer cells (Table 3). Activated parasporal proteins of both *B. thuringiensis* strains J61 and J72 were non-toxic (the degree of cytotoxicity is less than 5%) to Hs27 cells. However, activated parasporal proteins of J61 strain exhibited high selective cytotoxicity against MCF-7 cells and very high selective cytotoxicity against CACO-2, K562, Hep2, and HepG2 cells (Table 3). Whereas, activated parasporal proteins of J72 strain exhibited high selective cytotoxicity against K562 and Hep2 cells and very high selective cytotoxicity against CACO-2, MCF-7, and HepG2 cells. It was observed that the shape of parasporal proteins of strains J61 and J72 was spherical (Figure 1).

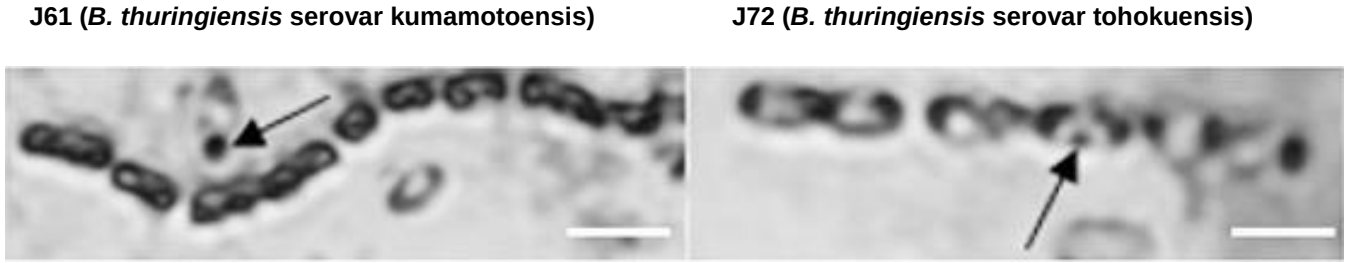

Figure 1. Microscopic photographs of parasporal proteins produced by *B. thuringiensis* serovar *kumamotoensis* strain J61 and serovar tohokuensis strain J72 stained with carbol-fuchsin stain. Scale bar is 5 µm. Black arrow heads show parasporal proteins.

Table 4. The inhibitory concentration (IC_{50}) of non-hemolytic proteinase K-activated parasporal proteins produced by *Bacillus thuringiensis* strains against five cancer cell lines.

***B. thuringiensis* strain**	***B. thuringiensis* serovar**	**IC_{50}[a] (µg/ml)**				
		CACO-2	**MCF-7**	**K562**	**Hep2**	**HepG2**
J61	*kumamotoensis*	0.70 (0.411-0.983)	1.00 (0.963-1.029)	1.98 (1.880-2.082)	2.36 (1.954-2.770)	1.27 (1.202-1.336)
J72	*tohokuensis*	2.55 (2.48-2.620)	2.79 (2.343-3.241)	3.63 (3.382-3.872)	3.25 (3.097-3.407)	3.32 (3.018-3.620)

[a]IC_{50}, The median inhibitory concentration. The confidence limit at the 95% level is given between parentheses.

As shown in Table 4, the IC_{50} values of the cytotoxic parasporal proteins against cancer cells were determined. Based on IC_{50} values, it was clearly observed that all cancer cells investigated in this study were more susceptible to activated parasporal proteins of serovar *kumamotoensis* strain J61 than that of serovar *tohokuensis* strain J72. Furthermore, CACO-2 and MCF-7 cells were the most sensitive to activated parasporal proteins of strains J61 and J72.

DISCUSSION

Cancer is a leading cause of death worldwide (nearly 1 in 6 deaths is due to cancer), accounting for 8.8 million deaths in 2015 according to the World Health Organization (WHO, 2017). The most common causes of cancer death and the deadliest are cancers of the lung (19.20% of deaths), liver (8.95%), colorectal (8.80%), stomach (8.57%), and breast (6.49%). About 70% of cancer deaths occur in low- and middle-income countries (WHO, 2017). As reported by the Ministry of Health (MOH, 2013), the cancer incidence in Jordan is in proportional increase. In addition, colorectal cancers and breast cancer are ranked first among Jordanian males and females, respectively. Therefore, the current study was established to investigate the anti-cancer activity of parasporal proteins produced by local *B. thuringiensis* strains against various human cancer cell types, in particular colorectal adenocarcinoma colorectal CACO-2 cells and breast cancer MCF-7 cells. Furthermore, the anti-cancer activity of *B. thuringiensis* parasporal proteins toward larynx epidermoid carcinoma Hep2 cells, liver hepatocellular carcinoma HepG2 cells, and leukemic K562T cells was examined.

Alkali solubilized and proteolytic processed parasporal proteins of the 92 *B. thuringiensis* strains used in this study were screened for their hemolytic activity against human erythrocytes. The hemolytic activity of parasporal proteins was measured before anti-cancer screening assay to ensure that the anti-cancer activity was not induced by hemolysins such as Cyt proteins that exhibited cytolytic activity against human

erythrocytes (Crickmore et al*.,* 1998, 2016). DTT-solubilized proteinase K-activated parasporal proteins of 58 *B. thuringiensis* strains exhibited either partial (α-hemolysis) or complete (β-hemolysis) patterns of hemolysis suggesting that cytolysins (known as Cyt proteins) or other hemolytic agents produced by those *B. thuringiensis* strains were responsible for hemolysis of erythrocytes. It was observed that activated parasporal proteins produced by the remaining 34 *B. thuringiensis* strains were non-hemolytic. Out of those 34 *B. thuringiensis* strains, nine strains were found to be non-toxic to human non-cancerous Hs27 cells. Of the non-toxic strains to Hs27 cells, only two local *B. thuringiensis* strains J61 (*B. thuringiensis* serovar *kumamotoensis*) and J72 (*B. thuringiensis* serovar *tohokuensis*) exhibited selective *in vitro* cytotoxicity against various human cancer cells (CACO-2, MCF-7, K562, HepG2, and Hep2). The activity in these strains was not attributable to the broad-spectrum Cyt proteins in view of the fact that they showed no hemolytic activity against human erythrocytes. Furthermore, the parasporal proteins of these *B. thuringiensis* strains (J61 and J72) were previously reported (Obeidat et al., 2012) to exhibit no insecticidal activity against diptera and lepidoptera. This is in agreement with previous studies (Mizuki et al*.,* 1999b, 2000; Lee et al*.,* 2000; Yamashita et al*.,* 2000; Ito et al*.*, 2004; Okumura et al*.*, 2004; Katayama et al*.,* 2005; Hayakawa et al*.*, 2007; Jung et al*.*, 2007; Uemori et al*.*, 2008; Yasutake et al*.*, 2008; Nagamatsu et al*.*, 2010) that clearly demonstrated that only non-insecticidal parasporal proteins can exhibit discriminating cytotoxicity against different cancer cell types. Unfortunately, activated parasporal proteins from all reference *B. thuringiensis* strains screened in this study did not exhibit selective cytotoxicity against target cells.

Mizuki et al. (1999b) found that the non-hemolytic leukemia cytotoxic strains belonged to serovars *dakota, neoleonensis, shandongiensis, coreanensis* and other unidentified serogroups. In a recent study, Ekino et al. (2014) reported that parasporal proteins produced from *B. thuringiensis* serovar *tohokuensis* were also cytotoxic against human leukemic T cells. Similarly, in a preceding study, Obeidat (2008) demonstrated that activated parasporal proteins of *B. thuringiensis* serovar *tohokuensis* exhibited selective cytotoxicity against larynx epidermoid carcinoma Hep2 cells and leukemic T cells, Jurkat. In this study, the non-hemolytic selectively cytotoxic *B. thuringiensis* strains were found to belong to serovar *kumamotoensis* and serovar *tohokuensis*. This observation demonstrated that cytotoxic proteins which exhibited specific cytotoxicity against cancer cells can be found in a variety of *B. thuringiensis* serovars. To our knowledge, no previous study has revealed the production of selective anti-cancer activity from processed parasporal proteins produced by *B. thuringiensis* serovar *kumamotoensis*.

According to IC_{50} values, protease-activated parasporal proteins of the cytotoxic strain J61 showed higher significant toxicity against cancer cells compared to that of strain J72. The 95% confidence limits of the IC_{50} values determined from MTT assay were not overlapped suggesting that J61 and J72 cytotoxicities are significantly different. Therefore, the cancer cells were more sensitive to activated parasporal proteins produced by strain J61.

It also appeared from the results that there is a marked variation among parasporal proteins in the level of cell-killing activity. Of particular interest is the fact that the activated parasporal proteins from strains J61 and J72 were able to discriminate between cancerous cells and noncancerous Hs27 cells, killing the former cells specifically. This finding strongly suggests the possible occurrence of *B. thuringiensis* strains in local habitats of Jordan which naturally produce parasporal proteins highly selective for cancer cells. This may lead to the use of these *B. thuringiensis* parasporal proteins for medical and pharmaceutical purposes including the treatment of particular types of cancer cells. Further studies regarding the identification of anti-cancer protein structure found in promising *B. thuringiensis* crudes are in process.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Correlation and path coefficient analysis of agronomic and quality traits in a bioenergy crop, sweet sorghum [*Sorghum bicolor* (L.) Moench]

Chalachew Endalamaw[1,2], Asfaw Adugna[3*] and Hussein Mohammed[2]

[1]Ethiopian Institute of Agricultural Research (EIAR), Melkassa Agriculture Research Center, P. O. Box 436 Adama, Ethiopia.
[2]Department of Plant and Horticultural Sciences, Hawassa University, P. O. Box 05, Hawassa, Ethiopia.
[3]Advanta Seeds Ltd., P. O. Box 10438, Eldoret, Kenya.

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Sweet sorghum is considered one of the best sources of bioethanol due to its higher total reducing sugar content, which ferments completely to produce ethanol coupled with its adaptation to the changing climate. A study was carried out in Ethiopia, during the 2015/16 crop season, to determine the extent of phenotypic and genotypic relationships among 13 agronomic traits and six quality components of 28 sweet sorghum genotypes. Panicle weight and width, dry matter yield, thousand kernel weight and harvest index had significant positive correlation with grain yield and exerted favourable direct effects both at phenotypic and genotypic levels. Ethanol yield was also correlated with juice yield, sugar yield and fresh stalk yield. Therefore, these yield and quality components are suggested to receive due attention during sweet sorghum varietal selection. Moreover, days to maturity had negative correlation and imposed negative direct effect on grain yield, which may indicate the possibility to select high yielding, early maturing dual purpose varieties for dry environments where terminal drought is rampant. The studied genotypes were grouped into three clusters according to their D^2 values, worthy of future breeding work considering the special merits in each cluster depending on the objectives of the breeding program. Some of the genotypes excelled as one of the two commercial sugarcane varieties used as controls for some quality traits. Therefore, considering their less water requirement, faster production cycle, and additional advantage of grain production over sugarcane, sweet sorghum stalks can serve as alternatives to sugarcane for use as feedstock in drier areas of the world under the changing climate.

Key words: Bioethanol, correlation, juice, path-coefficient, sweet sorghum.

INTRODUCTION

Sorghum's ability to withstand drought and heat stresses and to give reasonable yields under adverse

*Corresponding author. E-mail: asfaw123@rediffmail.com.

environmental conditions have raised its importance as a food security and bioenergy crop in arid and semi-arid tropics. In stress environments, pearl millet and sorghum are the dominant crops and receive fewer agricultural inputs than any other major cereals (McGuire, 2008). Sweet sorghum (*Sorghum bicolor*) is a natural variant of common grain sorghum with high stem sugar content, which can offer both food and fuel. The sugar content in the stalk juice of sweet sorghum reaches 10 to 25% at grain maturity (Pei et al., 2010). This sugar in the juice can be used to produce table sugar, syrup, wine or biofuel. The bagasse is used as forage or as raw material for the paper industry (Koeppen et al., 2009). Sweet sorghum ensures food and feed security and provides opportunities for additional income for small farmers serving as a feedstock for bioethanol production while protecting the environment (Almodare and Hadi, 2009). It requires 37% less nitrogen fertilizer and 17% less irrigation water than maize, and could yield more ethanol than maize during a dry year (Hills et al., 1990; Putnam et al., 1991). Its potential ethanol yield of 5000 L/ha/yr is more than that of sugarcane, maize, cassava and wood (Hodes, 2006).

There is an increasing interest in using sweet sorghum as source of bioethanol due to its various salient features including the higher total reducing sugar (glucose and fructose) and poor sucrose contents compared to sugarcane juice (Huligol et al. 2004), which prevent crystallization resulting in near complete fermentation efficiency to produce ethanol (Ratnavathi et al., 2004; Anderson, 2005). In general, alcohol as a fuel is clean, burning when used alone and when mixed with gasoline it acts to increase the octane rating (Schaffert and Gourley 1982), which may also mean that it contributes to climate change mitigation. Moreover, because of its efficient conversion of atmospheric CO_2 into sugar, sweet sorghum is a promising crop for use in the bio-energy industry and the ethanol production process from sweet sorghum is eco-friendly with less or no environmental pollution compared to that from molasses. However, information on the relationship of different agronomic and quality characters directly or indirectly involved in ethanol production is still meager. Therefore, the present study was carried out: to quantify the genetic correlations among various morpho-agronomic and quality traits in sweet sorghum genotypes; to identify the grain yield and bioethanol production potential among the genotypes; and to partition the correlation coefficients of various traits into direct and indirect effects.

MATERIALS AND METHODS

Description of the study site

Melkassa Agricultural Research Center (MARC) is located in the central Rift Valley of Ethiopia at a distance of 115 km from the capital Addis Ababa and 16 km south east of Adama town. The site is placed at an altitude of 1500 m above sea level on geographical coordinates of 8° 30' latitude and 39° 21' longitude. The area receives mean annual rainfall of 763 mm and the mean maximum and minimum temperatures are 24.8 and 14.0°C, respectively. Agro-ecologically, the area is categorized as dry semiarid. The soil is a well-drained typical sandy loam Andosols with a pH of 8.0.

Treatments and experimental design

The treatments consist of 28 genotypes including 26 accessions of sweet sorghum introduced from ICRISAT and preserved at MARC and two released grain sorghum varieties (Meko and Gambella 1107) as standard check (Appendix Table S1). The experiment was laid out in a Randomized Complete Block Design with three replications with each plot having 4 rows of 5 m length with row spacing of 0.75 m. Sowing was done by hand drilling on 4 July, 2015. Twenty days after planting (DAP), the seedlings were thinned to 0.15 m distance between plants. Phosphorus and nitrogen fertilizers were applied at the recommended rates of 100 and 50 kg-ha^{-1} in the form of DAP (46%P_2O_5, 18%N) and Urea (46%N), respectively. The DAP was applied during planting in the seed furrows with all plots top-dressed with urea when the plants reached 30 cm height. The experiment was conducted under rain fed conditions. Moreover, two commercial sugarcane varieties, [NCO-334(Cip) and B52298 (Wonji-1) from Wonji Sugar Corporation Estate of Ethiopia were used for comparison.

Data recording and analysis

Agronomic characteristics

Data were recorded on days to 50% flowering (DTF), days to maturity (DTM), plant height (PH) (cm), stalk diameter (cm) (STD), number of productive tillers (NPT), panicle length (PL) and width (PW), panicle weight (PWT), fresh stalk yield (FSY), dry stalk yield (DSY), thousand kernel weight (TKW) (at 12% moisture) and grain yield (GY). Stalk diameter was recorded as the average width of the middle part of the stem from five randomly selected plants in a plot at maturity using vernier caliper and record in centimeters. Productive tillers were recorded as the number of tillers that bear grain recorded from five randomly taken plants at maturity. Fresh stalk yield (kg) was measured from the two central rows of 25 randomly selected plants in each plot before harvesting for juice extraction. Dry stalk yield (kg) was measured from the two central rows of five randomly selected plants in each plot after.

Quality characteristics

The juice was extracted from 25 plants randomly taken from the two central rows in each plot and the volume was measured at hard dough stage. The juice was extracted using roller mills at Wonji Sugar Corporation Estate of Ethiopia. Due to the relatively long distance from the trial site to the extracting machine, the sample juice volume was expected to be biased. Therefore, alternative method of Wortmann et al. (2010) was followed to adjust the lost juice and sugar yields as follows:

JY (80% extracted) = [FSY – (DSY – CSY)] × 0.8;

CSY = (FSY – DSY) × Brix × 0.75;

SY=JY × Brix × 0.75;

Where, JY = juice yield (t ha^{-1}), FSY = fresh stalk yield (t ha^{-1}); DSY = dry stalk yield (t ha^{-1}); CSY = conservative sugar yield (t ha^{-1}) and SY = sugar yield (t ha^{-1}).

Brix % (BRX) was measured in the field from five randomly selected plants at middle portions of the stem from two central rows using refractometer (Atago 2522; Atago USA Inc., Bellevue, WA). To measure pol percent (POL), 200 ml of juice was transferred into a 300-ml Erlenmeyer flask, after purification with dry lead (Hornes dry lead) through filter paper No. 42. The pol tube was filled with the filtrate juice, and the POL reading was recorded from the Saccharimeter. Purity percent of the juice (PTY) was computed as (POL/BRX)100. Sugar yield estimates were calculated following the approach of Wortmann et al. (2010) that assumes 75% of the BRX as fermentable sugars. Theoretical EY ($L \cdot ha^{-1}$) was calculated from extracted juice as SY ($kg \cdot ha^{-1}$) multiplied by a conversion factor (0.581 L kg^{-1} ethanol) (Teetor et al., 2011). The above ground parts of five plants were chopped and kept in an oven at 70°C for 72 h to get dry stalk yield (DSY). Moreover, harvest index (HI) was calculated using the formula of Donald (1968) and expressed as percent.

Ten full canes were randomly collected from each of the two commercial sugarcane varieties from two rows. The plants were 12 months old during the time of sampling and their FSY, JY, BRX, POL, PTY, SY and EY were recorded to compare with the sweet sorghums.

Statistical analysis

The recorded data were subjected to analysis of variance using the procedures outlined by Gomez and Gomez (1984) using the GLM and PROC MIXED procedures implemented in SAS software v. 9.1.3 (SAS Institute 2003) and Program Genes (Cruz, 2006). Correlations among each pair of characters were also computed. The paired D-square value was computed based on the pooled mean of the genotypes and cluster analysis was obtained following the techniques of Tocher's (Rao, 1952).

RESULTS

Phenotypic and genotypic correlations among agronomic characters

Analysis of variance revealed highly significant differences among the genotypes for all the measured agronomic characters, except for number of tillers, indicating the existence of considerable genetic variability (data not shown). The comparative performance of sweet sorghum genotypes for agronomic traits is presented in Table 1. Phenotypic and genotypic correlations among agronomic characters are also presented in Appendix Table S2. Panicle weight, PW, HI, TKW and DMY were observed to have positive and significant correlations with GY at phenotypic and genetic levels, showing the inter-relationship of these traits. This was further confirmed by Path coefficient analysis (Appendix Tables S3 and S4). On the other hand, GY had significant negative correlation with DTM. Dry matter yield had significant positive correlation with DTF, PH, DSY and GY. Panicle weight and DMY had the highest positive direct effect on GY at phenotypic level, but only PWT and PL had the same effect at the genetic level. Days to maturity had negative direct effect on GY at both levels and their indirect effect via other characters was also mostly negative; thus, the relationship was mainly due to both direct and indirect effects.

Correlations among quality characters

Analysis of variance also revealed highly significant differences among the genotypes for all the measured quality characters (data not shown). The comparative performance of the studied genotypes for quality traits are presented in Appendix Table S5. Phenotypic and genotypic correlations among the quality characters are presented in Table 2. Juice yield and SY were observed to have positive significant ($p<0.01$) correlations with EY at phenotypic and genotypic levels. Ethanol yield had positive correlation with JY and FSY, but its correlation with the rest of the characters was not significant. Moreover, JY was highly correlated with SY. Plants with greater FSY and JY also produced greater SY and EY. Accordingly, genotypes with higher FSW produced higher JY that can be immediately fermented to bioethanol. Sweet sorghum genotypes those had the highest SY and EY were due to increased juice and high and moderate BRX, but they had moderate GY. Genotypes with high and moderate BRX, and high JY produced high SY and EY, and moderate GY. Brix percentage was significantly ($p<0.01$) correlated with POL at both levels, but it had no significant associations with other characters. The phenotypic and genotypic direct and indirect effects of different characters on EY are presented in Appendix Tables S6 and S7, respectively. Six of the nine characters studied showed positive direct effects on EY, whereas juice yield had the highest direct effect on EY followed by BRX.

Correlation among agronomic and quality characters

Wide genetic variability was found among the 28 genotypes for FSY, BRX, JY, SY and EY. Also, there were significant positive correlations among EY, FSY, and JY at phenotypic and genotypic levels, but the direct effect of FSY on EY was negligible. Phenotypic and genotypic path analysis showed that DTF, PH, BRX and POL had positive direct effect, but DTM, SW and DSY had negative direct effect and phenotypic and genotypic correlation with EY. Because their indirect effect via other characters was negligible, their phenotypic and genotypic correlation with EY was mainly due to direct effect.

Genetic divergence and cluster mean analysis

D-square analyses grouped the genotypes into three major clusters (Table 3), which may indicate that the tested genotypes were moderately divergent. The largest cluster (Cluster II) comprised of 16 genotypes (57.14%). Eight genotypes were grouped in Cluster III (28.57%) and the remaining four genotypes were included in Cluster I (12.29%). Cluster I was characterized by the highest PH, STD, DSY, DMY, POL, BRX and PTY, whereas Cluster II was characterized by the highest PWT, GY and HI. On

Table 1. Mean values of the 13 morpho-agronomic characters of the 28 genotypes studied at Melkassa during 2015/16.

Genotype	DF	DM	PH	PW	SWD	PL	PWT	FSY	DSY	GY	DMY	TKW	HI
104GRD	69.67	109.67	2.31	6.56	1.86	20.87	0.16	22.29	13.11	4.86	21.85	23.14	22.21
89MW 5073	60.33	105.33	1.79	8.28	1.83	22.53	0.23	17.40	9.56	8.90	22.37	30.41	39.84
E 36-1	59.00	107.00	1.61	7.51	1.51	22.07	0.21	16.96	7.56	7.40	19.52	36.74	38.33
Ent. # 64DTN	67.33	108.33	1.51	7.65	1.82	21.20	0.21	17.82	7.33	5.56	16.84	29.54	33.41
Gambella 1107	68.00	111.33	1.79	7.29	1.90	20.33	0.22	21.18	11.33	7.23	23.81	30.83	30.40
ICSB 324	69.00	113.67	1.70	4.81	2.19	25.93	0.14	18.58	13.30	5.37	22.81	25.35	23.91
ICSB 654	50.67	112.00	1.67	5.97	1.63	25.73	0.19	12.56	8.22	6.31	17.44	35.96	39.22
ICSR 93034	69.33	111.67	1.68	5.19	1.99	21.87	0.18	17.16	9.95	6.79	24.04	31.67	29.04
ICSV 700	68.67	116.33	2.25	4.87	2.05	18.07	0.22	26.00	22.89	6.51	32.96	29.02	19.83
ICSV 93046	62.67	106.33	1.73	8.07	1.96	21.73	0.18	19.20	9.04	6.61	20.37	30.12	33.55
IESV 91104 DL	67.67	106.33	1.79	7.86	1.82	19.73	0.24	22.89	13.83	8.43	27.69	28.07	30.82
IESV 92001 DL	65.00	107.67	1.75	9.19	2.08	19.93	0.21	20.27	12.56	8.29	25.67	34.35	32.50
IESV 92008 DL	65.00	106.00	1.64	8.80	1.63	21.20	0.21	14.44	8.89	7.18	20.63	33.89	34.99
IESV 92021 DL	67.00	110.00	1.60	8.09	1.71	19.73	0.19	20.56	10.59	6.22	21.30	27.99	31.70
IESV 92028 DL	66.33	112.67	1.75	7.00	1.77	21.40	0.21	23.78	11.74	6.81	22.37	31.05	30.57
IESV 92089 DL	61.00	106.00	1.65	7.79	1.80	21.00	0.19	14.64	7.96	7.31	19.74	29.99	38.05
IESV 92099 DL	61.67	107.67	1.69	7.91	2.11	21.20	0.19	16.22	8.37	6.28	17.93	33.80	34.86
IESV 92165DL	62.00	111.33	1.74	6.37	1.83	23.33	0.22	18.29	10.30	7.66	22.37	27.26	35.18
IESV 92207 DL	66.67	112.00	1.79	6.10	1.81	22.53	0.19	22.11	15.19	7.66	28.09	34.70	27.35
IS 2331	63.33	107.67	2.17	10.39	1.84	22.33	0.14	20.42	14.59	6.24	24.89	33.65	25.99
Kari Mtama 1	63.33	104.33	1.48	9.56	1.92	20.47	0.20	14.87	9.59	7.82	24.89	28.13	31.84
IESV 92022 DL	58.00	107.33	1.83	10.65	1.80	20.33	0.20	13.16	5.39	7.92	24.19	32.12	39.42
Meko	51.00	104.67	1.62	8.12	1.62	23.00	0.18	12.49	5.07	6.67	15.59	36.99	44.27
MR#22 X IS- 8613/1/2/5-2-1	69.33	112.67	1.60	6.63	1.89	19.13	0.11	14.11	5.67	4.22	13.87	23.54	30.99
MR #22 X IS- 8613/2/3-1-3	70.67	112.00	1.46	4.74	1.59	18.93	0.20	14.64	7.48	5.68	18.15	25.23	34.17
NTJ 2	62.67	105.00	1.74	8.57	1.88	20.33	0.18	19.60	8.74	7.16	20.93	33.17	34.22
S 35	59.33	104.00	1.58	8.32	1.80	20.13	0.17	17.71	6.22	6.57	16.59	33.69	40.27
SDSL 90167	66.33	108.33	1.74	9.14	1.89	19.00	0.22	22.62	9.93	7.30	22.04	27.76	33.63
Mean	63.96	108.83	1.74	7.55	1.84	21.22	0.19	18.28	10.16	6.82	21.75	30.65	32.88
LSD (5%)	9.0	12.5	0.5	5.7	0.4	4.3	0.1	9.1	7.8	2.2	14.9	9.9	23.9
CV%	4.4	3.6	8.1	23.5	6.3	6.4	15.0	15.6	24.1	10.1	21.4	10.1	22.8

**, * Significant at 1 and 5% probability levels, respectively.
DF= Days to 50% flowering, DM= Days to maturity, PHT= Plant height (m), PWD=Panicle width (cm), SD= Stalk diameter (cm), PL= Panicle length (cm), PWT= Panicle weight (kg), GY= grain yield (ton ha^{-1}), DMY= Dry matter yield (ton ha^{-1}), TSW= 1000 seed weight (g), HI= Harvest index.

the other hand, Cluster III was characterized by the highest JY, SY, and EY (Appendix Tables S6 and S7).

DISCUSSION

Phenotypic and genotypic correlations among agronomic characters

Similar to the present study, Tesso et al. (2011) reported that GY was positively associated with TKW, PW and PW among 200 sorghum accessions included in their studies. In the present study, GY had negative correlation with DTM at phenotypic and genotypic levels, which was in agreement with the results of Patted et al. (2011). Furthermore, DTM had negative direct effect on GY at both levels, and their effect via other characters was also mostly negative. These negative correlations may help to select early maturing genotypes with high grain yield for moisture stressed areas where terminal drought is recurrent. Moreover DTF was negatively correlated with such characters as PL, TKW and HI, which was similar to the results of Gaikwad et al. (2013) and Sowmy et al. (2015). Tesso et al. (2011) also reported significant negative correlation between TKW and DTF. Thousand kernel weight was positively correlated with PW and HI. Panicle width and PL had the highest positive direct effect on GY at genetic level, which shows that the correlation explained the true relationship and suggests that direct selection for these traits could be effective. Meanwhile, similar results were previously reported by Sowmy et al. (2015). The phenotypic and genetic residual

Table 2. Mean quality characters of sweet sorghum genotypes studied at Melkassa in 2015/16.

Genotype	JY	SY	EY	BRX	POL	PUR
104GRD	11.11	1.48	860.78	17.86	13.17	73.74
89MW5073	7.03	0.85	493.75	16.13	7.27	45.00
E36-1	9.62	1.24	721.08	17.15	10.87	63.01
Ent.#64DTN	9.44	1.18	688.47	16.85	10.86	64.43
Gambella 1107	11.36	1.28	744.81	15.10	10.36	68.83
ICSB324	6.30	0.84	487.48	17.72	14.57	82.44
ICSB654	5.70	0.77	448.23	18.05	13.17	73.06
ICSR93034	8.38	0.97	565.28	15.44	9.23	59.99
ICSV700	3.20	0.40	232.66	16.62	11.42	68.64
ICSV93046	7.51	0.85	496.38	15.17	8.91	59.04
IESV91104DL	10.60	1.30	752.65	16.16	9.87	61.03
IESV92001DL	5.59	0.73	426.89	17.53	7.02	40.13
IESV92008DL	5.04	0.68	393.47	17.95	11.89	66.17
IESV92021DL	7.11	0.73	425.10	13.71	8.09	58.91
IESV92028DL	10.82	1.34	778.14	16.50	10.05	60.88
IESV92089DL	8.38	0.84	489.37	13.40	6.94	51.78
IESV92099DL	5.52	0.72	416.25	17.45	11.70	67.44
IESV92165DL	4.74	0.58	336.82	16.31	11.41	69.84
IESV92207DL	8.41	1.05	608.32	16.61	8.17	49.38
IS2331	6.86	0.80	466.61	15.67	10.54	67.26
Kari Mtama1	4.72	0.56	323.97	15.71	10.95	69.70
IESV92022DL	5.66	0.64	374.53	15.18	11.04	72.75
Meko	8.32	0.89	519.77	14.38	8.04	55.87
MR#22XIS-8613/1/2/5-2-1	8.79	0.99	573.43	14.92	11.69	78.15
MR#22XIS-8613/2/3-1-3	6.42	0.77	448.38	16.03	11.11	69.49
NTJ2	11.39	1.54	892.17	17.97	14.01	77.94
S35	11.70	1.39	808.58	15.88	12.72	80.09
SDSL90167	12.39	1.50	870.31	16.08	10.14	63.17
Mean	7.93	0.96	558.70	16.20	10.54	64.94
LSD (5%)	4.7	0.6	350.9	2.2	5.6	33.4
CV%	18.5	19.7	19.6	4.3	16.6	16.1

**, * Significant at 1 and 5% probability levels, respectively.

value (0.218 and 0.04, respectively) showed that the characters in the path coefficient analysis accounted for 78.2 and 96% of the variation in GY at phenotypic and genetic levels, respectively (Appendix Tables S3 and S4). The positive associations among GY with PW and PW, TKW and DMY indicate that selecting for positively associated panicle related traits would have a positive effect on GY. Negative correlations were observed among some traits which could be utilized in breeding for negatively correlated traits.

Correlation among quality characters

The significant ($p<0.01$) correlations of JY and SY with EY in this study was in agreement with the results of Makanda et al. (2009) and Rutto et al. (2013) and may indicate the usefulness of these characters to improve EY. Thus, breeding for higher juice type genotypes might result in higher SY and EY than other traits. Brix was found to have no direct contribution to EY, which was against Gaikwad et al. (2013). Generally, correlation analyses indicated greater contribution of JY to higher SY and EY than BRX alone suggesting that improvement for high SY and EY could be achieved through selecting genotypes with high JY. Given the same BRX value, genotypes with greater JY produced higher sugar and ethanol yields (Table 2). Similar to this result, Makanda et al. (2009) reported that genotypes with higher JY and lower BRX had better SY than those genotypes with lower JY and higher BRX. The highest performing genotypes in the present study also confirmed that JY is an important trait for selection of higher SY and EY. Juice yield, which had positive and highly significant phenotypic correlation ($r = 0.970^{**}$) with EY had also the highest direct effect at phenotypic and genotypic levels, which

Table 3. Distribution of 28 sweet sorghum genotypes in different clusters.

Cluster	Number of genotypes	Genotypes
Cluster –I	4	104GRD, ICSB324, ICSV700, MR#22 × IS8613/1/2/5-2-1
Cluster-II	16	89MW5073, ICSB654, ICSR93034, ICSV93046, IESV92001DL, IESV92008DL, IESV92021DL, IESV92089DL, IESV92099DL, IESV92165DL, IESV92207DL, IS2331, Kari Mtama 1, IESV92022DL, Meko, MR #22 X IS- 8613/2/3-1-3, MR #22×IS- 8613/2/3-1-3
Cluster-III	8	E36-1, Ent. # 64DTN, Gambella 1107, IESV91104 DL, IESV92028 DL, NTJ2, S35, SDSL90167

was in agreement with the results of Shinde et al. (2013).

Correlation among agronomic and quality characters

The positive correlation between EY and FSY, and EY and JY at phenotypic and genotypic levels may indicate that sweet sorghum genotypes with improved SY and EY can be utilized for genetic improvement. The correlation between EY and FSY was in agreement with the findings of Alhajturki et al. (2014). Prasad et al. (2013) also reported significant correlations among EY, FSY, SY and JY. The positive correlation (r = 0.35*) of FSY with EY but negligible direct effect at both levels, may indicate that high FSY with high JY is a pre-requisite for high ethanol recovery (Rani and Umakanth, 2012). Hence, these traits could be utilized in the sweet sorghum breeding program. Critical analysis of character association and path analysis suggests that more focus needs to be given in selection programs for traits such as BRX, FSY, and JY. Phenotypic and genotypic path analysis showed that DTF, PH, BRX and POL had positive direct effect and phenotypic and genotypic correlation, but DTM, SW and DSY had negative values with EY. Because their indirect effect via other characters was negligible, their phenotypic and genotypic correlation with EY was mainly due to direct effect. The JY could also be directly related to FSY. Previous studies in sweet sorghum showed that FSY was correlated with PH, SD and JY (Audilakshmi et al., 2010) which it entails. Therefore, selection for FSY needs to take into consideration PH, SD, BRX and JY. Significant genotypic variability among sweet sorghum germplasm was also reported by Ali et al. (2008) and Murray et al. (2009) for PH and juice BRX.

D-square and cluster mean analyses

It is essential to determine how the influential traits lead to an improved sweet sorghum cultivar. The present study showed significant variation among the genotypes for the traits considered. Improvement in EY and GY could be achieved by direct or indirect selection for high yielding genotypes and for yield components positively associated with these target traits. Genotypes were grouped into three clusters and the future breeding program utilizing the studied accessions is suggested to be based on the genetic analysis of the various traits to which clusters are predominant. Hence, for future breeding work it could be useful to select individual genotypes from these clusters by considering the special advantages of each cluster and the objectives of the breeding program.

Advantages of sweet sorghum over commercial sugarcane as sources of bioethanol

Among the 28 genotypes, seven (NTJ 2, SDSL 90167, 104GRD, E36-1, Ent.#64DTN, IESV 92028 DL and S 35) had sugar-rich juice (JY, SY and EY), comparable to one of the commercial sugarcane varieties, NCO334(Cip) but the other commercial sugarcane variety B52298(Wonji-1) was superior in all characters (Appendix Table S8). Sweet sorghum genotypes were harvested in less than four months of growth period at MARC, while sugarcane varieties were 12 months old at the time of sample collection. Sweet sorghum genotypes were grown with less rainfall, whereas sugarcane varieties used all available rain and a large amount of irrigation water. Previous reports by Soltani and Almodares (1994) showed that sweet sorghum grown for ethanol production in India took about four months and water requirement of 8000 m^3 over two cropping seasons, which was four times less than those of sugarcane (12 to 16 months and 36,000 m^3 $crop^{-1}$, respectively). Similarly, it has been shown that the cost of cultivation of sweet sorghum is three times less than that of sugarcane (Dayakar Rao et al., 2004). In this study, sugarcane varieties had higher pol (sucrose), PTY, FSY and BRX than sweet sorghum genotypes (Appendix Table S9). However, in addition to sweet-stalk, which can be sold out to the distillers, grain yield of 4.22 to 8.9 t ha^{-1} is an added advantage of sweet sorghum over sugarcane, which can be used as food or for sale by the small holder farmer.

Conclusion

Those yield and quality components, which were significantly correlated in these study are suggested to receive due attention during sweet sorghum varietal

selection. The study has also shown the possibility of selecting high yielding, early maturing varieties for dry environments where terminal drought is rampant. Considering their less water requirement, faster production cycle, and additional advantage of grain production over sugarcane, sweet sorghums can serve as very good alternatives to sugarcane for use as feedstock to ethanol distillers in the drier areas of the world under the changing climate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIX

Table S1. List of the 28 Sweet sorghum genotypes used in the evaluation study at Melkassa in 2015.

SN	Genotype	Source
1	ICSV 93046	ICRISAT
2	IESV 92008 DL	ICRISAT
3	Kari Mtama 1	ICRISAT
4	NTJ 2	ICRISAT
5	IESV 91104 DL	ICRISAT
6	104GRD	ICRISAT
7	IESV 92165DL	ICRISAT
8	MR #22 X IS 8613/2/3-1-3	ICRISAT
9	ICSR 93034	ICRISAT
10	MR # 22 X IS 8613/1/2/5-2-1	ICRISAT
11	S 35	ICRISAT
12	IESV 92028 DL	ICRISAT
13	ICSB 654	ICRISAT
14	ICSB 324	ICRISAT
15	ICSV 700	ICRISAT
16	IESV 92022 DL	ICRISAT
17	IS 2331	ICRISAT
18	IESV 92021 DL	ICRISAT
19	IESV 92001 DL	ICRISAT
20	Ent. # 64DTN	ICRISAT
21	SDSL 90167	ICRISAT
22	E 36-1	ICRISAT
23	89MW 5073	ICRISAT
24	IESV 92099 DL	ICRISAT
25	IESV 92089 DL	ICRISAT
26	IESV 92207 DL	ICRISAT
27	Gambella 1107	Released variety
28	Meko	Released variety

Table S2. Phenotypic (above diagonal) and genotypic (below diagonal) correlation coefficients among 11 agronomic characters from 28 sweet sorghum genotypes.

Trait	DF	DM	PHT	PWD	SD	PL	PWT	GY	DMY	TSW	HI
DF	1	0.48**	0.19	-0.36*	0.40*	-0.46**	-0.07	-0.3	0.36*	-0.68**	-0.77**
DM	0.59**	1	0.28	-0.76**	0.23	0.06	-0.15	-0.43*	0.28	-0.38*	-0.62**
PHT	0.23	0.48**	1	-0.02	0.3	-0.08	-0.09	-0.06	0.58**	-0.12	-0.61**
PWD	-0.44*	-0.87**	-0.11	1	-0.09	-0.21	0.12	0.43*	-0.03	0.34*	0.39*
SD	0.51**	0.32	0.39*	-0.1	1	-0.04	-0.18	-0.07	0.38*	-0.3	-0.54**
PL	-0.54**	0.03	-0.15	-0.23	-0.02	1	-0.2	-0.02	-0.18	0.3	0.16
PWT	-0.08	-0.14	-0.22	0.16	-0.25	-0.3	1	0.73**	0.38*	0.17	0.24
GY	-0.32	-0.61**	-0.09	0.50**	-0.12	0.02	0.79**	1	0.48**	0.43*	0.35*
DMY	0.55**	0.59**	0.79**	-0.01	0.43*	-0.23	0.52**	0.61**	1	-0.05	-0.61**
TSW	-0.79**	-0.58**	-0.22	0.38*	-0.41*	0.37*	0.09	0.40*	-0.11	1	0.49**
HI	-1.00**	-1.00**	-1.00**	0.63**	-0.79**	0.28	0.28	0.47**	-0.37*	0.76**	1

**, *: Significant at 1 and 5% probability levels, respectively. DF= Days to 50% flowering; DM= days to maturity; PHT= plant height (m); PWD=panicle width (cm); SD= stalk diameter (cm); PL= panicle length (cm); PWT= panicle weight (kg); GY= grain yield (ton ha^{-1}); DMY= dry matter yield (ton ha^{-1}); TSW= 1000 seed weight (g); HI= harvest index.

Table S3. Estimates of direct (bold diagonal) and indirect effect (off diagonal) at phenotypic level of ten traits on grain yield in 28 sweet sorghum genotypes tested at MARC.

Trait	DF	DM	PHT	PWD	SD	PL	PWT	DMY	TSW	HI	r_p
DF	**0.406**	-0.077	0.002	-0.025	0.024	-0.103	-0.009	0.353	-0.044	-0.821	**-0.298**
DM	0.196	**-0.160**	0.003	-0.053	0.013	0.014	-0.020	0.277	-0.024	-0.666	**-0.435***
PHT	0.075	-0.044	**0.009**	-0.001	0.018	-0.017	-0.013	0.566	-0.008	-0.654	**-0.060**
PWD	-0.147	0.122	0.000	**0.070**	-0.005	-0.047	0.017	-0.033	0.022	0.421	**0.430***
SD	0.164	-0.036	0.003	-0.006	**0.058**	-0.010	-0.025	0.374	-0.019	-0.575	**-0.071**
PL	-0.187	-0.010	-0.001	-0.014	-0.003	**0.224**	-0.028	-0.174	0.019	0.167	**-0.019**
PWT	-0.027	0.023	-0.001	0.008	-0.011	-0.045	**0.138**	0.368	0.011	0.252	**0.727****
DMY	0.146	-0.045	0.005	-0.002	0.022	-0.040	0.052	**0.982**	-0.003	-0.651	**0.475****
TSW	-0.277	0.060	-0.001	0.023	-0.018	0.067	0.024	-0.045	**0.065**	0.528	**0.427***
HI	-0.312	0.100	-0.006	0.027	-0.031	0.035	0.033	-0.597	0.032	**1.071**	**0.352***

Residual= 0.218. DF= Days to 50% flowering; DM= days to maturity; PHT= plant height (m); PWD=panicle width (cm); SD= stalk diameter (cm); PL= panicle length (cm); PWT= panicle weight (kg); GY= grain yield (ton ha^{-1}); DMY= dry matter yield (ton ha^{-1}); TSW= 1000 seed weight (g); HI= harvest index.

Table S4. Estimates of direct (bold diagonal) and indirect effect (off diagonal) at genotypic level of ten traits on grain yield in 28 sweet sorghum genotypes tested at Melkassa (2015).

Trait	DF	DM	PHT	PWD	SD	PL	PWT	DMY	TSW	HI	r_g
DF	**0.605**	-0.240	-0.011	-0.126	-0.101	-0.505	-0.057	0.119	-0.048	0.064	**-0.315**
DM	0.358	**-0.405**	-0.023	-0.252	-0.064	0.032	-0.100	0.128	-0.035	0.070	**-0.612****
PHT	0.137	-0.192	**-0.048**	-0.032	-0.078	-0.141	-0.159	0.172	-0.013	0.052	**-0.092**
PWD	-0.265	0.354	0.005	**0.288**	0.021	-0.219	0.115	-0.002	0.023	-0.031	**0.500****
SD	0.307	-0.130	-0.019	-0.030	**-0.200**	-0.018	-0.178	0.094	-0.025	0.039	**-0.120**
PL	-0.324	-0.014	0.007	-0.067	0.004	**0.941**	-0.215	-0.051	0.022	-0.014	**0.017**
PWT	-0.049	0.057	0.011	0.046	0.050	-0.284	**0.712**	0.113	0.006	-0.014	**0.789****
DMY	0.332	-0.240	-0.038	-0.003	-0.087	-0.221	0.372	**0.216**	-0.006	0.018	**0.612****
TSW	-0.477	0.234	0.010	0.110	0.082	0.344	0.065	-0.023	**0.061**	-0.037	**0.404***
HI	-0.793	0.579	0.051	0.182	0.157	0.259	0.200	-0.079	0.047	**-0.049**	**0.474****

Residual =0.04. DF= Days to 50% flowering; DM= days to maturity; PHT= plant height (m); PWD=panicle width (cm); SD= stalk diameter (cm); PL= panicle length (cm); PWT= panicle weight (kg); GY= grain yield (ton ha^{-1}); DMY= dry matter yield (ton ha^{-1}); TSW= 1000 seed weight (g); HI= harvest index.

Table S5. Phenotypic (above diagonal) and genotypic (below diagonal) correlation coefficients among eight quality characters from 28 sweet sorghum genotypes.

Trait	FSY	DSY	JY	SY	EY	BRX	POL	PUR
FSY	1	0.80**	0.3	0.35*	0.35*	0.16	-0.06	-0.14
DSY	0.93**	1	-0.21	-0.15	-0.15	0.24	-0.01	-0.12
JY	0.31	-0.2	1	0.97**	0.97**	-0.08	0.04	0.08
SY	0.35*	-0.13	0.97**	1	1.00**	0.16	0.18	0.14
EY	0.35*	-0.13	0.97**	1.00**	1	0.16	0.18	0.14
BRX	0.16	0.26	-0.09	0.15	0.15	1	0.59**	0.24
POL	-0.15	-0.02	0.01	0.18	0.18	0.67**	1	0.92**
PUR	-0.29	-0.17	0.05	0.14	0.14	0.32	0.92**	1

**, *: Significant at 1 and 5% probability levels, respectively. FSY= Fresh stalk yield (ton ha^{-1}); DST= Dry stalk yield (ton ha^{-1}); JUY= Juice yield (ton ha^{-1}); SUY=Sugar yield (ton ha^{-1}); ETY = Ethanol yield (L ha^{-1}); BRX= Brix%; POL= POL juice.

Table S6. Estimates of direct (bold diagonal) and indirect effect (off diagonal) at phenotypic level of nine traits on ethanol yield in 28 sweet sorghum genotypes tested at Melkassa.

Trait	DTF	DTM	PH	SD	FSY	DSY	JY	BRX	POL	r_p
DTF	**0.016**	-0.010	0.004	-0.007	0.022	-0.017	0.135	0.011	0.001	**0.155**
DTM	0.008	**-0.021**	0.005	-0.004	0.014	-0.019	-0.198	0.030	0.004	**-0.181**
PH	0.003	-0.006	**0.020**	-0.005	0.023	-0.026	-0.030	0.045	0.002	**0.027**
SD	0.006	-0.005	0.006	**-0.016**	0.015	-0.016	-0.152	0.033	0.002	**-0.128**
FSY	0.009	-0.007	0.012	-0.006	**0.040**	-0.029	0.292	0.037	-0.001	**0.346***
DSY	0.008	-0.011	0.014	-0.007	0.032	**-0.037**	-0.200	0.054	0.000	**-0.147**
JY	0.002	0.004	-0.001	0.003	0.012	0.008	**0.959**	-0.018	0.001	**0.970****
BRX	0.001	-0.003	0.004	-0.002	0.006	-0.009	-0.077	**0.226**	0.010	**0.157**
POL	0.001	-0.004	0.002	-0.002	-0.002	0.000	0.035	0.134	**0.017**	**0.182**

Residual = 0.049. DF= Days to 50% flowering; DM= Days to maturity; PHT= plant height (m); SD= stalk diameter (cm); FSY= fresh stalk yield (ton ha^{-1}); DST= dry stalk yield (ton ha^{-1}); JUY= juice yield (ton ha^{-1}); SUY=sugar yield (ton ha^{-1}); BRX= Brix%; POL= Pol % juice.

Table S7. Estimates of direct (bold diagonal) and indirect effect (off diagonal) at genotypic level of nine traits on ethanol yield in 28 sweet sorghum genotypes tested at Melkassa Agricultural Research Center (2015/2016).

Trait	DTF	DTM	PH	SD	FSY	DSY	JY	BRX	POL	r_g
DTF	**0.059**	-0.044	0.006	-0.014	-0.049	0.048	0.179	0.020	0.002	**0.207**
DTM	0.035	**-0.074**	0.012	-0.009	-0.037	0.063	-0.310	0.062	0.006	**-0.252**
PH	0.013	-0.035	**0.024**	-0.011	-0.044	0.069	-0.034	0.053	0.003	**0.040**
SD	0.030	-0.024	0.010	**-0.028**	-0.031	0.039	-0.191	0.032	0.004	**-0.160**
FSY	0.042	-0.040	0.016	-0.013	**-0.069**	0.079	0.305	0.036	-0.003	**0.354***
DSY	0.033	-0.054	0.020	-0.013	-0.064	**0.085**	-0.197	0.059	0.000	**-0.130**
JY	0.011	0.023	-0.001	0.005	-0.021	-0.017	**0.990**	-0.020	0.000	**0.970****
BRX	0.005	-0.020	0.006	-0.004	-0.011	0.022	-0.086	**0.225**	0.015	**0.152**
POL	0.007	-0.021	0.003	-0.004	0.010	-0.002	0.009	0.151	**0.022**	**0.176**

Residual = 0.030. DF= Days to 50% flowering; DM= Days to maturity; PHT= Plant height (m); SD= Stalk diameter (cm); FSY= Fresh stalk yield (ton ha^{-1}); DST= Dry stalk yield (ton ha^{-1}); JUY= Juice yield (ton ha^{-1}); SUY=Sugar yield (ton ha^{-1}); BRX= Brix%; POL= Pol % juice.

Table S8. The summary of cluster means of 19 quantitative traits for the sweet sorghum genotypes based on data set.

Characters	Cluster means			
	I	II	III	Overall Mean
Days to 50% flowering	69.17	62.35	64.58	65.37
Days to maturity	113.09	108.25	107.87	109.74
Plant height	1.97	1.71	1.69	1.79
Panicle width	5.72	7.83	7.92	7.16
Stem diameter	2.00	1.82	1.80	1.87
Panicle length	21.00	21.62	20.52	21.05
Panicle weight	0.16	0.19	0.21	0.19
Fresh stalk yield	20.25	16.78	20.32	19.12
Dry stalk yield	13.74	9.55	9.59	10.96
Grain yield	5.24	7.10	7.06	6.47
Juice yield	7.35	6.59	10.92	8.29
Sugar yield	0.93	0.78	1.35	1.02
Ethanol yield	538.59	452.07	782.03	590.90
Brix	16.78	15.92	16.46	16.39
Pol	12.71	9.72	11.11	11.18
Purity	75.74	60.99	67.42	68.05
Dry matter yield	22.87	21.73	21.22	21.94
Thousand seed weight	25.26	31.64	31.36	29.42
Harvest index	24.24	34.50	33.96	30.90

Table S9. Yield in bio energy traits for 28 sweet sorghum genotypes and two commercial sugarcane varieties.

Sweet sorghum genotypes	FSY	JY	SY	EY	BRX	POL	PUR
104GRD	**3.34**	**1.25**	**0.16**	**0.1**	**17.86**	**13.17**	**73.74**
89MW 5073	2.61	1.06	0.13	0.07	16.13	7.27	45
E 36-1	**2.54**	**1.28**	**0.17**	**0.1**	**17.15**	**10.87**	**63.01**
Ent. # 64DTN	**2.67**	**1.42**	**0.18**	**0.1**	**16.85**	**10.86**	**64.43**
Gambella 1107	3.18	1.31	0.15	0.09	15.1	10.36	68.83
ICSB 324	2.79	0.72	0.1	0.06	17.72	14.57	82.44
ICSB 654	1.88	0.59	0.08	0.05	18.05	13.17	73.06
ICSR 93034	2.57	0.97	0.11	0.07	15.44	9.23	59.99
ICSV 700	3.9	0.29	0.04	0.02	16.62	11.42	68.64
ICSV 93046	2.88	1.36	0.15	0.09	15.17	8.91	59.04
IESV 91104 DL	3.43	1.22	0.15	0.09	16.16	9.87	61.03
IESV 92001 DL	3.04	1.05	0.14	0.08	17.53	7.02	40.13
IESV 92008 DL	2.17	0.76	0.1	0.06	17.95	11.89	66.17
IESV 92021 DL	3.08	1.32	0.14	0.08	13.71	8.09	58.91
IESV 92028 DL	**3.57**	**1.62**	**0.2**	**0.12**	**16.5**	**10.05**	**60.88**
IESV 92089 DL	2.2	0.88	0.09	0.05	13.4	6.94	51.78
IESV 92099 DL	2.43	1.06	0.14	0.08	17.45	11.7	67.44
IESV 92165DL	2.74	1.08	0.13	0.08	16.31	11.41	69.84
IESV 92207 DL	3.32	0.94	0.12	0.07	16.61	8.17	49.38
IS 2331	3.06	0.78	0.1	0.06	15.67	10.54	67.26
Kari Mtama 1	2.23	0.71	0.08	0.05	15.71	10.95	69.7
IESV 92022 DL	1.97	1.04	0.12	0.07	15.18	11.04	72.75
Meko	1.87	0.99	0.11	0.06	14.38	8.04	55.87
MR # 22 X IS 8613/1/2/5-2-1	2.12	1.13	0.13	0.07	14.92	11.69	78.15
MR #22 X IS 8613/2/3-1-3	2.2	0.96	0.12	0.07	16.03	11.11	69.49
NTJ 2	**2.94**	**1.48**	**0.2**	**0.12**	**17.97**	**14.01**	**77.94**
S 35	**2.66**	**1.54**	**0.18**	**0.11**	**15.88**	**12.72**	**80.09**
SDSL 90167	**3.39**	**1.71**	**0.21**	**0.12**	**16.08**	**10.14**	**63.17**
NCO-334(Cip)	**26**	**1.55**	**0.21**	**0.12**	**18.49**	**17.08**	**92.4**
B52/298(Wonji-1)	30	2.8	0.42	0.25	20.13	18.68	92.8

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A method of rapid *in vitro* proliferation and morphological characterization of the medicinal plant *Aloe vera* L.

Chandra Sekhar Singh B.[1*], Diriba Adugna A.[1] and Roja Rani A.[2]

[1]Department of Plant Science, College of Agriculture and Ambo University, Ambo, Post Box : 19, Ethiopia.
[2]Department of Genetics and Biotechnology, Osmania University, Hyderabad-500007, India.

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Morphological characterization was carried out on *Aloe vera* (*Aloe barbadensis* Miller) accessions collected from different locations in India. The experiments were conducted on 12 Aloe accessions and the leaf length, width, thickness, stem length, and thickness were measured using vernier calipers. The plantlets were chemically treated with 2.5% (w/v) of sodium hypochlorite which is an alternative safer surface sterilization method for replacement of mercury chloride by using shoot tip as explants. The sterilization results indicate that the highest number (93%) of viable explants (10) with the best regeneration potential was in Murashige and Skoog (MS) medium compared with other treatments. Multiple shoots/explants (35) were found in MS medium containing 6-benzylaminopurine (BAP, 1.5 mgL^{-1}; kinetin (0.5 mgL^{-1}), indole-3-butyric acid (IBA, 0.2 mg L^{-1}), indole-3-acetic acid (IAA, 0.1 mg L^{-1}), and naphthalene acetic acid (NAA, 0.2 mg L^{-1}). Based on the shoot proliferation, a maximum number (87.89%) of shoots obtained from the micro-shoot culture method and a maximum number (91.12%) of roots were obtained on MS medium supplemented with NAA (0.2 mg L^{-1}), IBA (0.2 mgL^{-1}) and IAA (0.1 mgL^{-1}) which was found to be the best. The healthy rooted plantlets were successfully transferred and obtained as the plants were of 100% survival. The regenerated plants were morphologically similar to the mother plant.

Key words: *Aloe vera*, morphology, micro propagation, sodium hypochlorite, shoot tips, efficient method, medicinal plant.

INTRODUCTION

Aloe vera L. (*Aloe barbadensis* Mill.) belonging to the family Liliaceae (Xanthorrhoeaceae) is an important perennial, xerophytic, medicinal, succulent herb and the genus comprises about 300 perennial species. Generally, *Aloe* species are identified by their leaf structure, characteristics, inflorescences and rosettes of succulent leaves (Singh et al., 2013). Aloe can grow in nutritionally poor soil and it has great demand in pharmaceutical and

*Corresponding author. E-mail: singhsekhar960@gmail.com. Tel: + 91 7619180292.

cosmetic industry. The plant has green fleshy leaves covered by a thick cuticle or rind and an inner clear pulp (Williams et al., 2010). Aloe is used externally for the treatment of burns, scalds, skin irritation, sunburn wounds, psoriasis, acne, dermatitis, eczema, and ulcers. The plant has numerous pharmacological values like antioxidant, anti-inflammatory, antidiabetic, anti-microbial, anti-fungal, anti-viral, anti-tumor, antiseptic, anti-aging, and other properties (Paoulomi et al., 2013). Administration of gel extract reduces blood glucose levels, blood urea and glycosylated haemoglobin (Rajasekaran et al., 2005).

In additional to the pharmaceutical applications, *Aloe vera* is widely used in food and cosmetic industry. In the food industry, it is used as an ingredient for health drinks, desserts and beverages and yogurt (Reynolds, 2004). In addition to the medical applications, *Aloe vera* has been introduced in cultivation as ornamental and house hold plant. The morphological studies are very important for cultivating the high quality of the Aloe gemplasm.

Aloe is generally propagated by suckers arising from the base of the mother plant and natural vegetative propagation of Aloe is very slow. Earlier studies showed that *Aloe vera* has been sterilized using the mercuric chloride which is harmful for the environment and also has effect on kidney, liver, adrenal and fertility (WHO, 2005). Mercury chloride ($HgCl_2$) is biotransformed into methyl mercury chloride (CH_3HgCl) and causes adequate environmental effects by human consumption of contaminated fish, shellfish, and algae (Silva-Pereira, 2005). The alternative method for rapid multiplication of selected genotypes is possible by using sodium hypochlorite, which is the main objective of the present study.

Tissue culture of different *Aloe* spp. was reported by many researchers. Cytokinins are one of the most important growth regulators affecting the shoot proliferation (Garland and Stolz, 1981). This research work which deals with the use of shoot tip and apical meristem for micro propagation have been proposed by Debiasi et al. (2007) and Campestrini et al. (2006). Acclimatization of rooted plantlets in pots containing a mixture of sand, silt and compost under greenhouse conditions with 80 to 90% of moisture is suited for young plants survival (Hirimburegama and Gamage, 1995). Currently, it is necessary to develop efficient regeneration method for Aloe cultivation to meet the high demand, particularly, for plant genetic transformation and cloning techniques (Velcheva et al., 2005). Micro propagation of various accessions of Aloe from different regions in India has not been reported so far. Several studies have reported about rapid *in vitro* propagation of *Aloe vera* (Albanyl, 2006). Regeneration of *Aloe vera* in nature is very slow due to its male sterility which forms a barrier in rapid propagation. Aloe is an exclusively propagated crop using lateral buds or off shoots produced by donor plant. A single plant usually produces 2 to 3 off shoots in a year which is not sufficient for undertaking commercial cultivation and to meet the industrial demand.

The main objective of this study was to develop a rapid, efficient, cost effective, and easy method of micropropagation of *Aloe vera* at commercial level. In this study, media composition with new combinations and concentrations of different growth regulators for efficient and rapid micro propagation of *Aloe vera* was standardized using shoot tip explants.

MATERIALS AND METHODS

Collection of ex-plant material

This study was carried out on germplasm of *Aloe vera* (*A. barbadensis* Miller), an important medicinal and ornamental plant collected from different places such as National Bureau of Plant Genetic Resources (NBPGR), New Delhi and Central Institute of Medicinal and Aromatic Plants (CIMAP), Boduppal, Hyderabad, India. The experimental plant material consists of 12 accessions of *Aloe vera (A. barbadensis* Miller); among them, 10 accessions were collected from NBPGR, voucher no: NBPGR/ 2011/1771 dated 11-07-2011 from different geographical regions of India from Rajasthan (IC111267, IC111269, IC111271, IC111272), Gujarat (IC111279), Haryana (IC111280), and Delhi (IC471882, IC471883, IC471884, IC471885), respectively and 2 accessions of *Aloe* CIM-Sheetal (CAL 14) and wild *Aloe vera* (local) were collected from CIMAP, voucher no: CIMAP/63/6222 dated 20-07-2011. All the plants used in this study were about two years of age. High potential accessions showed the highest incidence in morphological studies which are as shown in Figure 1a to f and the details of 12 accessions grown at Indian Immunologicals Ltd. Shown in Table 1.

All the accessions were examined for a number of morphological parameters; from each accession, 5 mature plants were randomly selected and the observations were recorded and mean data were used for statistical analysis. Morphological data of 12 individuals were observed. The data was transformed into numerical form and was analyzed by Newman-Kuel's multiple comparisons test. While studying *Aloe* genus, various characters such as number of leaves, number of suckers, leaf width/breadth (cm), stem length (cm), peduncle length (mm), leaf thickness (density of leaf) (mm), gel fresh weight (g), leaf dry weight (g), biomass (g), root length (cm), and Aloin concentration (%) were considered.

Sterilization of explants

The shoot apices were washed thoroughly under running tap water for 30 min to remove all the adhering dust particles and microbes from the surface. The explants were then washed with 0.1% of liquid detergent (Labolene, France) for another 30 min and then rinsed several times with distilled water to remove detergent. The explants were then treated with 1% of Bavistin (systemic fungicide) and 0.25% of Dithane M-45 for 30 min, and were washed with distilled water. Then, were later treated with ampicilin (systemic bactericide) along with streptomycin for 30 min each to eliminate fungus and bacteria and the fungicide and bactericide were washed out with sterilized distilled water. Under the sterile conditions, explants were rinsed with isopropyl alcohol for 45 s and then 10 explants were treated with 2.5% sodium hypochlorite (10% Clorox) for 30 min. The explants were then thoroughly washed (4 to 5 washings) with sterilized distilled water to remove the traces of sodium hypochlorite and were trimmed to remove extra outer portion of stem discs and carefully cultured in sterile culture bottles containing 40 ml of MS medium under laminar air flow hood

Figure 1. High potential accessions of *Aloe vera* shown for morphological and *in vitro* studies. (a) *Aloe barbadensis* Mill IC111271; (b) *Aloe barbadensis* Mill IC111272; (c) *Aloe barbadensis* Mill *Aloe* CIM-Sheetal; (d) *Aloe barbadensis* Mill IC111279; (e) *Aloe barbadensis* Mill IC471882; (f). *Aloe barbadensis* Mill *Aloe vera* (local).

Table 1. Details of *Aloe vera* accessions used for morphological and *in vitro* studies.

S/N	Species	Accession	Obtained from	Source
1	*Aloe barbadensis* Mill	IC 111267	Rajasthan	NBPGR, New Delhi
2	*Aloe barbadensis* Mill	IC 111269	Rajasthan	NBPGR, New Delhi
3	*Aloe barbadensis* Mill	IC 111271	Rajasthan	NBPGR, New Delhi
4	*Aloe barbadensis* Mill	IC 111272	Rajasthan	NBPGR, New Delhi
5	*Aloe barbadensis* Mill	IC 111279	Gujarat	NBPGR, New Delhi
6	*Aloe barbadensis* Mill	IC 111280	Haryana	NBPGR, New Delhi
7	*Aloe barbadensis* Mill	IC471882	Delhi	NBPGR
8	*Aloe barbadensis* Mill	IC471883	Delhi	NBPGR
9	*Aloe barbadensis* Mill	IC471884	Delhi	NBBPGR
10	*Aloe barbadensis* Mill	IC471885	Delhi	NBPGR
11	*Aloe barbadensis* Mill	*Aloe CIM-Sheetal* (CAL14)	Uttarpradesh	CIMAP, Hyderabad
12	*Aloe barbadensis* Mill	wild *Aloe vera* (local)	Telangana	CIMAP, Hyderabad

(Murashige and Skoog, 1962). After sterilization, shoot apices were directly inoculated into different concentrations of media for shoot elongation (Ahmed et al., 2007).

Culture medium

The basal medium contains Murashige and Skoog (MS) medium salts, vitamins, 3% sucrose and agar. The basal media was supplemented with various concentration and combination of growth regulators: 2, 4-D, 6-benzylaminopurine (BAP), naphthalene acetic acid (NAA), kinetin (KIN), indole-3-butric acid (IBA), and indole-3-acetic acid (IA). Sucrose (3%) was used as carbon source and media were solidified with agar (0.7%). Preparation of media ingredients and their concentrations as per MS medium are shown in Table 2. The medium was adjusted to pH 5.9 prior to autoclaving at 121°C and 15 lbs pressure for 20 min. The reagent grade chemicals were obtained from Fischer Scientifics and Hi Media

Table 2. Preparation of media ingredients and their concentrations as per Murashige and Skoogs.

Components	IM	MM-1	MM-2	MM-3	MM-4	RM-1	RM-2
KNO_3	1900	1900	1900	1900	1900	1900	1900
NH_4NO_3	1650	1650	1650	1650	1650	1650	1650
$CaNO_3$	-	-	-	-	-	-	-
$Cacl_2.\ 2H_2O$	440	440	440	440	440	440	440
$MgSO_4.7H_2\ O$	370	370	370	370	370	370	370
KH_2PO_4	170	170	170	170	170	170	170
$MnSO_4.4H_2O$	22.3	22.3	22.3	22.3	22.3	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6	8.6
$FeSO_4.7H_2O$	27.85	27.85	27.85	27.85	27.85	27.85	27.85
$Na_2.EDTA.2H_2O$	37.3	37.3	37.3	37.3	37.3	37.3	37.3
$COC1_2.6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25
H_3BO_3	6.2	6.2	6.2	6.2	6.2	6.2	6.2
KI	0.83	0.83	0.83	0.83	0.83	0.83	0.83
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1	0.1
PyridoxinHCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Meso inositol	100	100	100	100	100	100	100
Sucrose	30000	30000	30000	30000	30000	30000	30000
Agar	7000	7000	7000	7000	7000	7000	7000
Kinetin	-	-	0.5	0.5	0.5	-	-
NAA	-	0.1	0.2	0.2	0.2	-	0.2
IAA	0.3	0.1	0.1	0.1	0.1	-	0.1
IBA	-	0.1	0.2	0.2	0.2	-	0.5
BAP	2.5	2.0	1.5	1.0	0.5	-	-
PG	-	-	-	-	90	-	-
Ca.Pantothenate	-	-	-	-	100	-	-

IM, Initial Media; MM-1, MS medium-1; MM-2, MS medium-2; MM-3, MS medium-3; MM-4, MS medium-4; RM-1, MS rooting medium-1; RM-2, MS rooting medium-2.

Laboratories, India. The media sterilization was done before adding growth hormones into it because they are heat sensitive.

Inoculation of explants

After sterilization of explants, they were inoculated in culture bottles aseptically and explants were transferred onto sterile paper with the help of sterile forceps for inoculation under aseptic conditions. The explants were further trimmed and extra outer leaves were removed to make them in suitable sizes (2 to 3 cm). After cutting, explants were vertically inoculated into culture of 500 mL capacity bottles containing MS medium with 0.3 mgL^{-1} of IAA and 3 mgL^{-1} of BAP; then the bottles were tightly capped and properly sealed with wrap to avoid entry of external air. The bottles were transferred to growth chamber. All the cultures were kept in the culture room at 28 ± 1°C and at photoperiod of 16 h provided by cool-white fluorescent light and the cultures were observed periodically. Only root cultures were maintained in darkness.

Establishment of shoot cultures

Explants of *in vitro* regenerated plantlets were used for proliferation of shoot cultures on the basal MS medium supplemented with kinetin (0.5 mgL^{-1}) and BAP (1.0 mgL^{-1}) in combination with NAA (0.1 or 0.2 mgL^{-1}) and with IAA (0.1 mgL^{-1}) and IBA (0.2 mgL^{-1}) for shoot amplification. Shoots were multiplied by repetitive transfer of original explants. Cluster of shoots amplified from initial lateral shoot explants were sub-cultured as it is without separation from the explants on the same regeneration media after one month from initial establishment stage multiplication of shoots were replicated 3 times with 10 explants per treatment. Multi shoots were divided into single micro shoots and transferred to fresh medium every 5 weeks of subculture. The data was recorded after 30 days of culture and only shoots greater than 2 cm were considered for taking data. The percentage of shoot induction, total number of shoots and length of shoots were recorded after 6 weeks of fifth, seventh, and eighth subcultures. The percentage of shoot induction was calculated as the total number of initial explants, which gave response to hormonal treatment per total number of explants multiplied by 100%. After another 6 weeks of incubation, the proliferating cultures were transferred to different media for shoot elongation (Table 4).

Establishment of root cultures

The newly formed shoots were measured in the length of 2 to 3 cm,

excised individually from the parent explants and transferred to a rooting media. Two types of rooting medium were used; one is MS basal media with 3 types of hormones NAA, IAA, IBA and the other one is half strength MS media. The data was recorded after 30 days of culture.

Acclimatization

The crucial step of micro propagation is acclimatization of the *in vitro* obtained plantlets. *In vitro* regenerated plants of *Aloe vera* with no morphological abnormalities were transplanted into pots with a survival rate of 100%. After 30 days, the micro propagated plantlets with well-developed roots were successfully acclimatized to *ex vitro* conditions. The pots (8×6 cm) were kept ready for filling with garden soil, compost and sand in the proportion of 2:1:1, respectively. The plants were then transplanted into the pots, then thoroughly watered and kept under plastic house having 80% humidity and 31°C temperature for 10 days. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary, then the plants were shifted to shade house with less humidity and indirect sunlight. After 15 days, the plants were transferred to the soil. The micro propagated plants were morphologically uniform and grew well in the field. In the present study, about 100% of plantlets survived from tissue culture to the experimental plot, whereas Baksha et al. (2005) observed the survival rate of 70%, Dwivedi et al. (2014) observed 83% survival for Indian Aloe and Liao et al. (2004) observed 93% survival for Chinese Aloe.

Statistical analysis

The collected data were subjected to one way analysis of variance (ANOVA) carried out in different accessions of *Aloe vera* covering various parameters followed by Newman-Kuel's multiple comparisons test. The ANOVA revealed highly significant differences among various accessions for various characters. The results indicated the presence of adequate amount of genetic variability in the germplasm.

RESULTS AND DISCUSSION

Morphological studies

The evaluation and characterization of germplasm is prerequisite for any breeding programme aimed at improvement of yield. The qualitative as well as quantitative evaluation of germplasm is not only conducive in core collection, but also for its utilization in cultivation and breeding. The assessment of variability existing in the germplasm of accessions is of great interest for conservation of genetic resources and also for broadening of genetic base of species to be exploited by plant breeders. It helps in systematic cultivation of the plant for commercial purposes. The morphological studies for *Aloe* spp. are very essential characteristics for the medicinally and economically important genus to regularize the commercial and economical production.

In the present study, 12 Aloe accessions were studied and the morphological characteristics results are shown in Table 3. The result shows the occurrence of variation in morphological and biochemical characteristics when compared with the mother plants and their tissue culture generated plants. The extent of variation, however, differs from accession to accession. This could be related to differences in the genotypes of various accessions. Morphological analysis indicates that all the studied characteristics have a significant difference at $P < 0.01$ among Aloe accessions. Based on the morphology, the collection of accessions of *Aloe vera* germplasm has been divided into two morphotypes that are small *Aloe vera* (SAV) (plant size up to 40 cm) and large *Aloe vera* (LAV) (plant size above 40 cm). The LAV type of accessions shows the highest incidence. Accession IC111272(4), IC471883 (8), IC471882 (7), IC111267(1), and *Aloe* CIM-Sheetal (11) were found to be the tallest (61 to 67 cm range) as it possessed a distinct stem (calescent) with long inter nodes. Minimum leaf thickness (18.1 mm) for accession IC111271(3) and minimum wideness (3.9 cm) accession for local *Aloe vera* (12) was recorded. Leaf dry weight of local *Aloe vera* (12) was very low (12.20 g) and the highest (23.7 g) was in IC 111269 (2) among other accessions. Gel fresh weight was recorded as 58.52 ml/100 g in IC 111267 (1) with overall mean value of 20.025 as the highest and the lowest was 4.10 g in local *Aloe vera* (12) though the fresh gel weight was highest in IC 111267(1) and the dry gel weight was recorded as 12.80 g in IC111271 (3) as compared to all other accessions. Lowest aloine concentration recorded about was 0.86% from IC111267 (1), whereas the highest was 1.68% for accession IC111280 (6).

The medicinal and cosmetic value of the Aloe depends on the quantity of its biochemical constituents. Presently, an attempt has been made to evaluate the collected *Aloe vera* germplasm for its quality and yield components. The quality of the germplasm depends on the relative composition of leaves/leaflet. The plants producing higher amount of fresh gel, dry gel, biomass, presence of chemical compounds and lower amount of aloin are consider being of good quality. The accessions of IC111271, IC111269, IC111267 and *Aloe* CIM-Sheetal *s*how better performance *in vitro* and *in vivo* than the local *Aloe vera* (wild) obtained from Hyderabad region. Among the present studies of Aloe accessions, the leaf yield per plant ranged between 0.652 kg and 23.2486 kg/plant and dry leaf weight between 12.20 and 23.70 g/kg. Accessions with high yield are IC111271, IC111269, IC111267 and *Aloe* CIM-Sheetal. The amount of gel (dry) obtained from one plant in one year is estimated to be 2.10 to 12.80 g/plant. High yield of gel can be obtained from accessions IC111271, IC111269 and *Aloe* CIM-Sheetal. Morphochemical evaluations can provide insight into the genetic structure and diversity within and among varieties from different geographical origins, producers and distributors. Without this information, there is no means of selecting appropriate plant material for the participation in screening programs with a view to introduce the novel varieties for industrial purpose (Radhamadhavi et al., 2012).

Table 3. Morphological studies of Aloe accessions.

S/N	Accession number	Number of leaves	Number of suckers/plant	Peduncle length	Leaf length (cm)	Leaf width (cm)	Stem length (cm)	Leaf weight (g)	Leaf thickness (cm)
1	IC 111267	13.0 ± 0.3^{b}	3.0 ± 0.2^{c}	88.0 ± 0.5^{h}	51.0 ± 0.4^{b}	4.0 ± 0.06^{e}	13.1 ± 0.3^{a}	353.3 ± 0.25^{j}	22.1 ± 0.04^{c}
2	IC 111269	12.0 ± 0.3^{c}	1.0 ± 0.1^{e}	124.0 ± 0.6^{c}	49.0 ± 0.4^{c}	4.8 ± 0.03^{d}	10.3 ± 0.2^{b}	548.5 ± 0.2^{c}	31.6 ± 0.04^{a}
3	IC 111271	12.0 ± 0.3^{c}	5.0 ± 0.04^{a}	183.0 ± 0.5^{a}	48.0 ± 0.4^{bc}	5.2 ± 0.05^{c}	11.3 ± 0.2^{ab}	483.3 ± 0.07^{d}	18.1 ± 0.06^{e}
4	IC 111272	15.3 ± 0.1^{a}	1.0 ± 0.1 ^{e}	118.0 ± 0.6^{d}	55.0 ± 0.4^{a}	6.1 ± 0.05^{a}	12.3 ± 0.1^{a}	561.6 ± 0.2^{b}	18.8 ± 0.06^{e}
5	IC 111279	12.0 ± 0.3^{c}	1.0 ± 0.1^{e}	83.0 ± 0.4^{hi}	49.0 ± 0.3^{c}	4.5 ± 0.03^{de}	10.6 ± 0.04^{b}	361.7 ± 0.7^{i}	20.3 ± 0.1^{d}
6	IC 111280	12.0 ± 0.3^{c}	4.0 ± 0.04^{b}	85.0 ± 0.4^{i}	51.0 ± 0.3^{b}	4.6 ± 0.05^{de}	8.5 ± 0.06^{d}	370.2 ± 0.2^{h}	19.5 ± 0.04^{de}
7	IC471882	13.3 ± 0.1^{b}	4.0 ± 0.04^{b}	108.0 ± 0.2^{f}	51.0 ± 0.4^{b}	5.9 ± 0.2^{ab}	12.1 ± 0.04^{a}	466.6 ± 0.2^{f}	18.5 ± 0.04^{de}
8	IC471883	13.2 ± 0.2^{b}	2.0 ± 0.05^{d}	115.0 ± 0.4^{e}	52.0 ± 0.4^{ab}	5.8 ± 0.05^{ab}	12.4 ± 0.06^{a}	691.7 ± 0.04^{a}	20.8 ± 0.06^{d}
9	IC471884	12.2 ± 0.1^{c}	2.0 ± 0.05^{d}	103.0 ± 0.6^{g}	48.0 ± 0.2^{bc}	6.1 ± 0.05^{a}	12.7 ± 0.06^{a}	406.7 ± 0.08^{g}	22.3 ± 0.05^{c}
10	IC471885	11.1 ± 0.1^{cd}	1.0 ± 0.1^{e}	88.0 ± 0.4^{h}	48.0 ± 0.2^{bc}	5.2 ± 0.05^{c}	11.3 ± 0.1^{ab}	333.3 ± 0.1^{k}	25.3 ± 0.06^{b}
11	*Aloe* CIM-Sheetal (CAL-14)	14.3 ± 0.1^{ab}	5.0 ± 0.05^{a}	143.0 ± 0.4^{b}	49.0 ± 0.2^{c}	5.6 ± 0.04^{b}	12.2 ± 0.1^{a}	471.6 ± 0.02^{e}	21.6 ± 0.09^{c}
12	Wild *Aloe vera* (Local)	10.2 ± 0.1^{d}	3.0 ± 0.06^{c}	78.0 ± 0.4^{j}	46.0 ± 0.3^{d}	3.9 ± 0.05^{e}	9.8 ± 0.06^{c}	342.6 ± 0.2l	20.3 ± 0.05^{d}

Values are means + SE. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Kuel's multiple comparisons test.

Table 3. Cont'd.

S/N	Accession number	Leaf dry weight (g)	Gel fresh weight (g)	Gel dry weight (g)	Biomass (g/plant)	Root length (cm)	Aloin concentration (%)
1	IC 111267	23.5 ± 0.02^{a}	58.52 ± 0.04^{a}	12.4± 0.05^{a}	683.2± 0.37^{c}	14.23± 0.1^{d}	0.86 ± 0.008^{e}
2	IC 111269	23.7 ± 0.03^{a}	56.27 ± 0.08^{b}	12.4± 0.05^{a}	713.2±0.12^{b}	2.32 ± 0.5^{e}	0.9 ± 0.007^{e}
3	IC 111271	20.35±0.05^{b}	49.98±0.03^{c}	12.8±0.03^{a}	720.8±0.4^{a}	23.91±0.1^{b}	0.91±0.007^{e}
4	IC 111272	19.9 ± 0.04^{b}	20.7±0.05^{d}	8.5±0.01^{b}	223.3±0.37^{d}	9.67±0.07^{ef}	1.15±0.005^{c}
5	IC 111279	19.7± 0.04^{b}	15.12±0.06^{e}	6.3±0.04^{c}	199.2±0.32^{e}	14.72±0.07^{d}	1.4±0.007^{b}
6	IC 111280	15.8± 0.02^{c}	8.85±0.03^{f}	3.8±0.05^{d}	123.2±0.38^{f}	15.34±0.1^{d}	1.68±0.001^{a}
7	IC 471882	15.6± 0.03^{c}	6.3±0.05^{g}	3.4±0.02^{d}	86.7±0.38^{g}	26.25±0.2^{a}	1.36±0.009^{b}
8	IC 471883	12.64±0.01^{d}	4.72±0.05^{h}	2.2±0.01^{e}	58.01±0.34^{j}	18.52±0.07^{c}	1.3±0.007^{b}
9	IC 471884	12.81±0.03^{d}	4.79±.01^{h}	2.2±0.05^{e}	58.4±0.19^{j}	14.12±0.1^{d}	1.08±0.003^{c}
10	IC 471885	13.8±0.08^{c}	5.3±0.06^{gh}	3.1±0.04^{d}	74.4±0.17^{h}	7.65±0.07^{f}	0.83±0.002^{d}
11	*Aloe* CIM-Sheetal	14.13±0.06^{c}	5.65±0.02^{gh}	3.2±0.04^{d}	70.28±0.1^{i}	27.34±0.07^{a}	0.78±0.004^{d}
*12	Wild *Aloe vera* (Local)	12.2±0.09^{d}	4.1±0.06^{h}	2.1±0.05^{e}	56.2±0.2^{j}	8.91±0.04^{ef}	1.2±0.007^{c}

Values are means + SE. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Kuel's multiple comparisons test.

Table 4. Effect of MS medium composition and Shoot proliferation response from shoot tips cultured on medium with different concentrations of growth regulators.

S/N	Growth regulator (mgL^{-1})	Response (%)	Average number of shoots/explant	Average length of shoots (cm)
1	IAA+ BAP (0.3 + 3.00)	18.79 ± 0.01^{e}	3.80 ± 0.05^{e}	2.60 ± 0.05^{e}
2	IBA+IAA+NAA+BAP (0.1+0.1+0.1 + 4.5)	27.84 ± 0.04^{d}	4.58 ± 0.03^{c}	3.18 ± 0.05^{c}
3	KIN+IBA+IAA+NAA+BAP (0.5+0.2+0.1+0.2+1.5)	87.89 ± 0.02^{a}	8.84 ± 0.03^{a}	4.89 ± 0.03^{a}
4	KIN+IBA+IAA+NAA+BAP+ GA_3 (0.5+0.2+0.1+0.2+1.5+0.5)	77.34 ± 0.08^{b}	6.71 ± 0.07^{b}	3.56 ± 0.02^{b}
5	KIN+IBA+IAA+NAA+BAP+GA_3 (0.5+0.2+0.1+0.2+1.5+1.0)	65.68 ± 0.05^{c}	4.23 ± 0.03^{d}	2.88 ± 0.01^{d}

Multiplication of shoots was replicated three times with 10 explants per treatment. Values are means±SE. Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

In vitro study

In the *in vitro* study, the shoot initiation, shoot multiplication, rooting and hardening of plantlets for 12 accessions were studied. The surface sterilization process is an important step to avoid any type of endogenous and exogenous contaminants and also standardized media composition for the mass multiplication and fast growth of *Aloe vera*. In the present investigation, shoot tip was used as an explants cultured on MS supplemented medium with various concentrations and combinations. Establishment of aseptic cultures was difficult, but once a healthy culture was established, there was no further contamination. Under given conditions and over a culture of 60 days, explants from all the treatments produced multiple explants and roots simultaneously. In earlier studies, *Aloe vera* has been sterilized using the mercuric chloride which is harmful for environment. The method of the present study is easy and new for explants sterilization for *in vitro* culture of *Aloe vera*.

The ability of plant cell, tissue and organs to differentiate plants in culture has resulted in widespread applications in propagation and plant breeding. Conventional method of propagation in *Aloe vera* is through vegetative means (side buds) which is very slow. There is no viable seed setting in the plants. Aloe is exclusively a vegetatively propagated crop where young side branches are used as planting material. Single plant may produce 2 to 3 side shoots per year making availability of planting material in good quantity and quality, a problem.

Keeping the aforementioned things in mind, tissue culture studies were undertaken in the *Aloe vera* plants in this study. A number of protocols have been developed for micropropagation of Aloe plants using a variety of explants like shoot tip, axillary bud, stem cuttings, etc., by various researchers (Hosseini and Parsa, 2007). The method used for clonal propagation is also an important tool for raising pathogen free plants in culture (Walkey, 1980). Tissue cultures of *A. barbadensis* were established by Sanchez et al. (1988) using vegetative meristems and leaf explants. Shoot tips and auxillary buds are popular explants for micropropagation of Aloe (Aggarwal and Barna, 2004).

Shoot initiation

For *in vitro* clonal propagation of *Aloe vera* plants, shoot tips were used as explants. Explants were obtained from healthy parent plants of 12 accessions for micro propagation. After sterilization, shoot apices were directly inoculated into various media. These explants were inoculated in wide mouth culture bottles containing MS medium. The media was supplemented with different concentrations of BA alone or in combination with IBA or IAA. The explants cultured in combination of MS basal medium with BA, NAA, KIN and IBA started showing signs of proliferation after 15 days of culturing (Figure 2a to f). Best growth was observed on MS medium supplemented with 1.5 mgL^{-1} BAP, 0.2 mgL^{-1} IBA, 0.1 mgL^{-1} IAA, 0.5 mgL^{-1} KIN, and 0.5 mgL^{-1} GA_3. New buds appeared from the axils of leaves of shoot explants and bud developed.

Cytokinin level produced a significant response upon the number of explants formed per plant and also showed influence on production of leaf numbers and rooting (Dwivedi et al., 2014). The shoot tip of explants initially produces 2 to 3 shoots within two weeks after inoculation. But in our method, 15 to 35 shoots/cultures were produced from single explant by subsequent 2 to 3 subcultures with the same medium which indicate the high efficiency of this protocol. The average length of shoots per culture was 4.89 ± 0.03 cm. Formation of the roots was best observed in *Aloe* CIM-Sheetal and IC111271 in the media containing MS basal media with 3 types of hormones NAA 0.2 mgL^{-1}, IAA 0.1 mgL^{-1} and IBA 0.2 mgL^{-1} within four weeks after inoculation for rooting. Proliferating shoots obtained from shoot tip explants of *Aloe* took maximum 6 to 7 weeks from the time of establishment to attain the size (2 to 3 cm) suitable for rooting. The highest percentage of shoots that induced roots (91.12%) was observed in MS medium

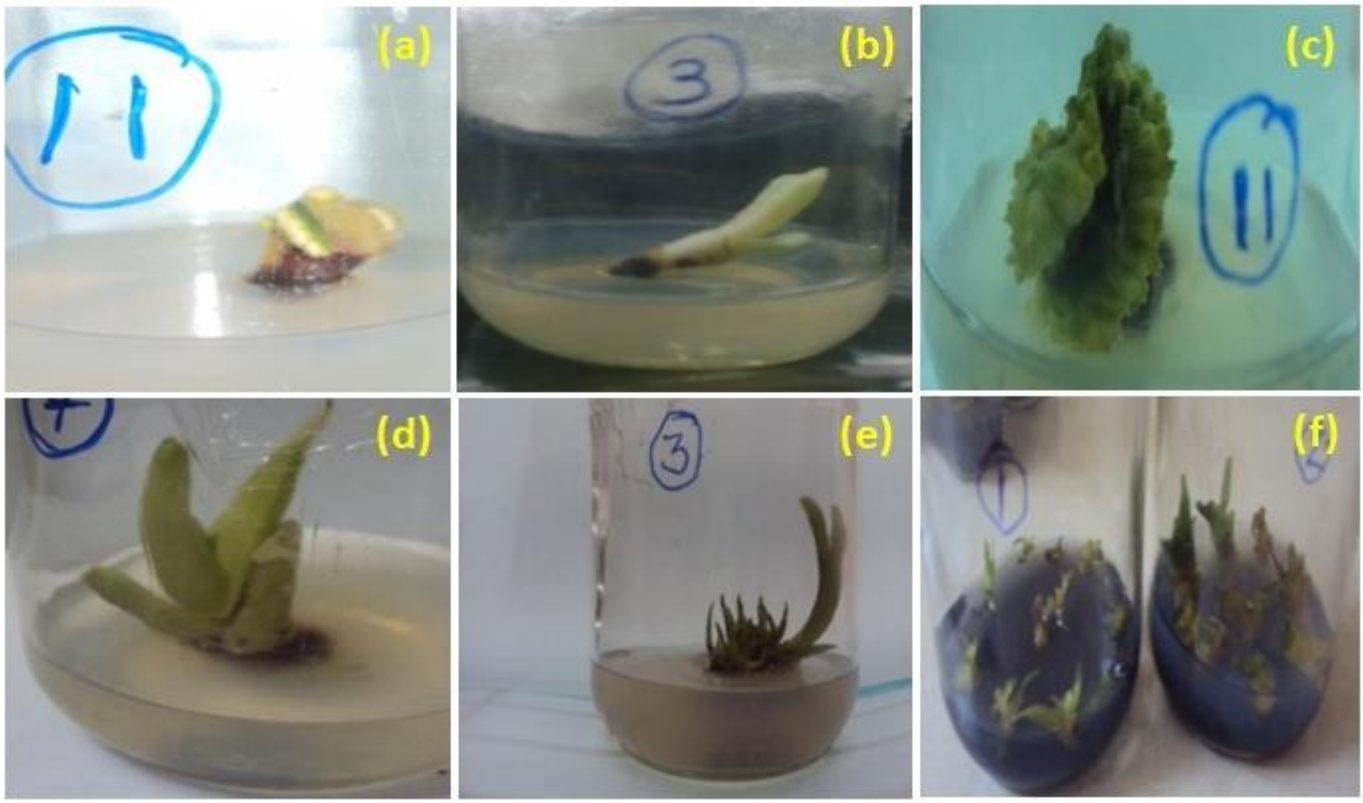

Figure 2. (a) Shoot tip regeneration in 1st week- *Aloe* CIM-Sheetal (11). (b) Shoot tip regeneration in 1st week-accession IC111271(3). (c) 30 Days regenerated shoot tip of accession IC471882 (7). (d) Regeneration of multiple shoots in *Aloe* CIM Sheetal (11). (e) Multiple shoots in early stage before subculture in IC111271(3). (f) Sub cultured shoot tips in IC111267(1) and IC111269(2).

supplemented with NAA (0.2 mgL^{-1}), IAA (0.1 mgL^{-1}) followed by IBA (0.2 mgL^{-1}).

Shoot multiplication

After initiation of the growth on the explants (30 days culturing), the newly formed shoots were excised individually with the help of sterilized blade and re-cultured on fresh bottles containing the same medium (MS medium with different supplements) to increase the number of shoots. The results obtained in the present study revealed that BAP at concentration of 1.5 mgL^{-1} provides better shoot multiplication. All the cultures showed shoot proliferation on MS medium with different concentrations. On an average, each explants produced 5 to 8 shoots (Figure 3a to l).

The results show that accessions *Aloe* CIM-Sheetal (Tag no:11), IC 111271 (3), IC 111279 (5), and IC111269 (2) are high potential accessions, IC 471882 (7), IC111267 (1), IC471885 (10) have shown moderate proliferation and IC 111280 (6), IC111280 (8), IC471884 (9), and wild *Aloe vera* (12) showed poor shoot proliferation and rooting among all accessions for their multiplication ratio of axillary buds, multiple shoots/clumps, and roots were regenerated after sub culturing. Cytokinins are one of the most important growth regulators affecting the shoot proliferation (Garland and Stolz, 1981). A range of cytokinins BA, kinetin and 2-ip have been used in micropropagation (Bhojwani and Razdan, 1992). BA variations affecting shoot proliferation were also reported by Bhandari et al. (2010), Gantait et al. (2010), Mangal (2009), and Chaudhuri and Mukundan (2001) also reported the use of BA in shoot proliferation of *Aloe polyphylla* and *A. vera*, respectively. Some researchers have shown that the presence of both auxin and cytokinin is necessary for shoot proliferation (Rout et al., 2001).

An interesting result was observed in explants preparation, sizing and a single mother plant can be multiplied for 30 to 35 explants per mother plant and 8.84 ± 0.03 shoots per explants were obtained on MS medium supplemented with BAP (1.5 mgL^{-1}), KIN (0.5 mgL^{-1}), NAA (0.2 mgL^{-1}), IAA (0.1 mgL^{-1}), and IBA (0.2 mgL^{-1}) in 7 weeks, in comparison with 30 shots from 18 explants obtained from 18 mother plants in 8 weeks and 20 shoots per plant in 8 weeks was reported (Balraj and Neelu, 2009). It was also reported that the enhancement of shoots was observed by using BA and NAA. The clusters of shoots were separated into pieces and each was sub-

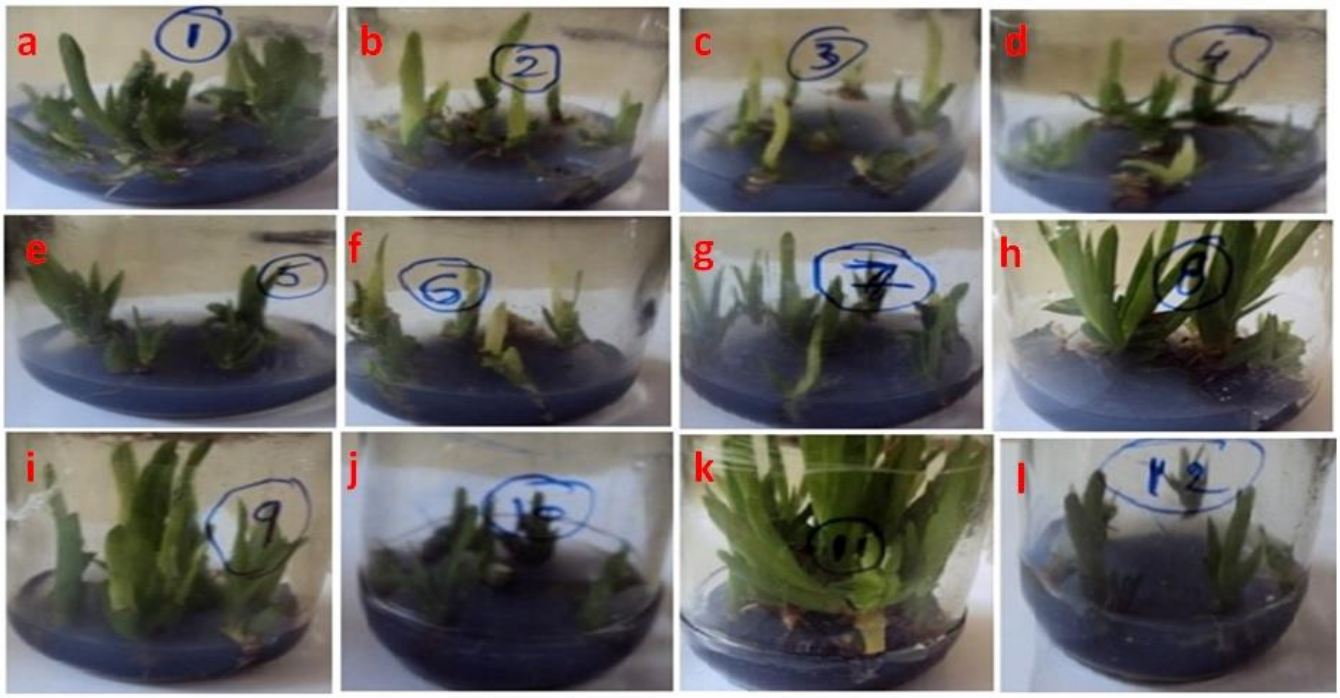

Figure 3. Regeneration of multiple shoots on MS medium of 12 accessions. (a) IC111267. (b) IC111269. (c) IC111271. (d) IC111272. (e) IC111279. (f) IC111280. (g) IC471882. (h) IC471883. (i) IC471884. (j) IC471885. (k) *Aloe* CIM-Sheetal (CAL14). (l) wild *Aloe vera* (local).

cultured individually on the same medium periodically. After third subculture, the shoot multiplication rate remained constant. On the other hand, regeneration of shoot buds was moderate (65 to 77%) on a medium containing: 1.5 mgL^{-1} BAP, 0.5 mgL^{-1} kinetin, 0.2 mgL^{-1} of IBA, 0.1 mgL^{-1} of IAA, 0.2 mgL^{-1} of NAA, and 1.5 mgL^{-1} BAP, 0.5 mgL^{-1} kinetin, 0.2 mgL^{-1} of IBA, 0.1 mgL^{-1} of IAA, 0.2 mgL^{-1} of NAA and 1.0 mgL^{-1} of GA_3, respectively. Comparatively, the lowest number of 18 to 28% adventitious shoots were observed in the medium containing 0.3 mgL^{-1} of IAA, 3.0 mgL^{-1} of BAP (IM) and 0.1 mgL^{-1} NAA, 0.1 mgL^{-1} of IBA, 0.1 mgL^{-1} of IAA, and 4.5 mgL^{-1} BAP. The highest concentration of 4.5 mgL^{-1} of BAP did not increase shoot proliferation. It was also reported that the highest shoot proliferation in *A. vera* was found in MS medium containing BA and IBA (Mukesh et al., 2011), where better proliferation occurred on medium containing kinetin instead of BA (Dwivedi, 2014). NAA and IBA are the most commonly used for root induction (Bhojwani and Razdan, 1992). Effect of 0.1 mgL^{-1} of NAA, 0.5 mgL^{-1} of IAA, and 0.5 mgL^{-1} of IBA on MS medium in rooting showed poor response.

For shoot proliferation, growth regulators especially cytokinins (Bhojwani, 1980) are one of the most important factors affecting the response. A range of cytokinin (kinetin, BA, 2-ip and Zeatin) has been used in micro propagation of work. Studies conducted by different researchers have clearly shown that BA is a more effective, reliable and useful cytokinin for shoot proliferation in *Aloe vera* (Debiasi et al., 2007). IBA (Chaudhuri and Mukundan, 2001) and acetic acid (Mukherjee and Roy, 2008) were also reported to be helpful in shoot proliferation in Aloe. Meyer and Staden (1991) reported auxillary shoot formation using IBA, whereas Roy and Sarkar (1991) obtained shoot on medium containing 2, 4-D with Kinetin. Richwine et al. (1995) reported induction of shoots using Zeatin. Liao et al. (2004) reported that the best medium for micro propagation of *Aloe vera* was MS + 2 mgL^{-1} BA + 0.3 mgL^{-1} NAA. Budhiani (2001) demonstrated that the best initiation and multiplication of shoot was on MS medium supplemented with 0.2 mgL^{-1} BAP + 0.002 mgL^{-1} NAA and 2 mgL^{-1} BAP + 0.002 mgL^{-1} NAA, respectively. Hashemabadi and Kaviani (2008) reported that MS with 0.5 mgL^{-1} BA and 0.5 mgL^{-1} NAA produced the highest number of shoots. According to Liu (2001), the best medium for shoot proliferation is MS with BA (1.0 mgL^{-1}) and IBA (0.3 mgL^{-1}). Best medium for bud initiation according to Liao et al. (2004) is MS with 2.0 mgL^{-1} BA + 0.3 mgL^{-1} NAA with 30 gL^{-1} sucrose + 0.6 gL^{-1} PVP (pH 5.8). Hirimburegama and Gamage (1995) cultured the plant on MS medium supplemented with 0.18 mgL^{-1} IAA and 2.25 mgL^{-1} BA. Zhou et al. (1999) suggested MS + 6 BAP (3 mgL^{-1}) as the best medium for the induction of buds. Also, in the present study, shoot proliferation occurred in the presence of cytokinin. The different

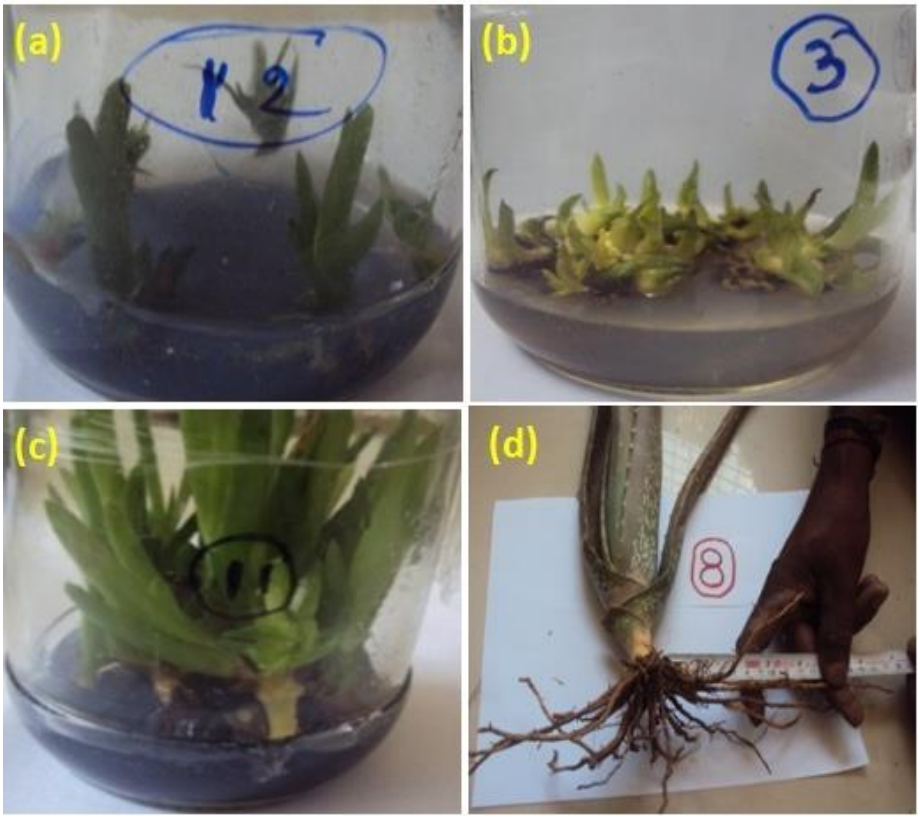

Figure 4. Shoot proliferation of various aloe accession on MS medium: (a) Regeneration of multiple shoots on MS+1.0 mgL^{-1} BAP+ 0.5 mgL^{-1} KIN+0.2 mgL^{-1} NAA + 0.1 mgL^{-1} IAA and 0.2 mgL^{-1} IBA in accession wild *Aloe vera* (12); (b) Multiple shoot regeneration after subculture of IC111271(3); (c) Root induction on MS + 0.2 mgL^{-1} NAA + 0.1 mgL^{-1} IAA + 0.2 mg IBA of *Aloe* CIM Sheetal (11); (d) Measurement of hardened plantlet root length of accession IC471883 (8).

phytohormonal combinations of BAP proved to be more effective. MS medium supplemented with 1.5 mgL^{-1} BAP + 0.2 mgL^{-1} IBA + 0.5 mgL^{-1} KIN + 0.1 mgL^{-1} IAA + 0.2 mgL^{-1} NAA + 0.5 mgL^{-1} GA_3 gave (77 to 88%) best shoot proliferation/multiplication. This is in contrast with earlier work by Meyer and Staden (1991) who reported better proliferation in *Aloe vera* on medium containing kinetin instead of BA. This difference may be due to the difference in the genotype of plant used (Abrie and Staden, 2001).

Rooting

The *in vitro* raised 3 to 4 cm long shoots were excised individually from the proliferated shoot clumps and cultured on rooting media where MS medium was supplemented with different concentrations of NAA. All the combinations showed induction of roots. Maximum number of roots (2 to 7) per plant was obtained in plantlets cultured on MS + 0.2 mgL^{-1} of NAA. The plantlets cultured on MS medium supplemented with 2 to 10 mgL^{-1} NAA showed induction of only one root per shoot. The roots obtained were creamish yellow in color and with/without branching. Newly formed micro roots measuring 2 to 3 cm in length (Figure 4a to d) were excised individually from the parent explants and transferred to rooting media. Two types of rooting media were used; one is MS basal media with 3 types of hormones NAA, IAA, IBA and other half strength MS media (Table 5). Data were recorded after 30 days of culture.

The highest root response in *A. vera* was reported in hormone free medium (Bhandari et al., 2010). In the current study, healthy rooting was observed in NAA (0.2 mgL^{-1}) and IBA (0.2 mgL^{-1}) medium. Healthy roots (number > 7 and length > 3 cm) were obtained in 8 weeks of time. Hardening is an important step in tissue culture. Rooting response of micro shoots is reported to be controlled by growth regulators in the medium (Abrie and Staden, 2001), basal salt composition (Garland and Stoltz, 1981), genotype (Rines and McCoy, 1981) as well as cultural conditions. NAA and IBA are the most commonly used auxins for root induction (Bhojwani and

Table 5. Effect of auxins on rooting of *Aloe vera* shoots after 6 weeks of In vitro culture.

Conc. of growth regulators (mgL^{-1}) in MS medium	percentage of shoots showing root formation	Average number of roots per shoot (mean ± SE)	Average length of root (cm) per shoot (mean ± SE)
0.1 NAA	65.57 ± 0.05[d]	4.98 ± 0.005[d]	2.09 ± 0.03[c]
0.2 NAA	91.12 ± 0.05[a]	6.89 ± 0.007[a]	3.08 ± 0.04[a]
0.5 NAA	79.87 ± 0.3[b]	6.15 ± 0.005[b]	2.97 ± 0.005[b]
0.1 IBA	49.83 ± 0.2[e]	2.98 ± 0.003[f]	1.88 ± 0.005[d]
0.2 IBA	74.93 ± 0.03[c]	5.51 ± 0.003[c]	2.91 ± 0.005[b]
0.5 IBA	40.64 ± 0.07[f]	2.75 ± 0.008[g]	2.04 ± 0.05[c]
0.1 IAA	33.65 ± 0.05[g]	3.65 ± 0.005[e]	0.97 ± 0.003[e]
0.2 IAA	26.72 ± 0.08[h]	1.31 ± 0.007[h]	0.84 ± 0.005[f]
0.5 IAA	-	-	-

The experiment was replicated two times with 10 explants per treatment. Values are means ± SE. Mean values followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

Razdani, 1992). By use of IBA, many plants such as *Lycoperscicon esculentum* (Sibi, 1982), *Hedychium roxburgii* (Tripathi and Bitaillion, 1995) and Carnation (Werker and Leshem, 1987) gave *in vitro* rooting. Rooting was achieved on MS medium + 0.18 mgL^{-1} NAA + 0.226 mgL^{-1} BA (Hirimburegama and Gamage, 1995). Zhou et al. (1999) used supplements NAA (0.3 mgL^{-1}) and IBA (0.3 mgL^{-1}) for rooting and found that NAA was better than IBA in the average number of roots produced and rooting rate. Best rooting was observed by Liao et al. (2004) by using ½ MS + 0.2 mg NAA.

The shoot tips subjected to MS medium supplemented with different concentration of NAA were also examined. Maximum rooting was observed in MS + 0.2 mgL^{-1} NAA + 0.2 mgL^{-1} IBA + 0.1 mgL^{-1} IAA. These results are in agreement with the results of Zhou et al. (1999). However, Natali et al. (1990), Meyer and Staden (1991), and Richwine et al. (1995) reported rooting in hormone free medium. In the present study, no rooting was obtained in hormone free medium even on prolonged waiting.

Regeneration of plants from callus may help to induce variability in the Aloe germplasm for future improvement. Over the last years, a number of micropropagation protocols have been developed using a variety of explants like shoot tips (Hashemabadi and Kaviani, 2008), auxillary buds (Hirimburegama and Gamage, 1995), stem cutting and leaf explants and plant regeneration via callus formation in *A. barbadensis* which occurs at low frequency (Sanchez et al., 1988). Successful establishment of calli and subsequent plantlet regeneration is reported in *Aloe pretoriensis* (Groenewald et al., 1975), *Aloe saponaria* (Yagi et al., 1983) and *A. vera* (Gui et al., 1990; Roy and Sarkar, 1991). Racchi (1987) used MS medium supplemented with 0.5 mgL^{-1} 2,4-D and 1 mgL^{-1} kinetin for root explants and 0.2 mgL^{-1} 2,4-D and 1 mgL^{-1} kinetin for leaf meristems. In *A. saponaria*, the best results were obtained using root tissue with a combination of 1 ppm indoleacetic acid (IAA) and 0.5 ppm 2,4-D and 2 ppm kinetin (Yagi et al., 1983). However, the occurrence of plant regeneration from these calli was not reported. Gui et al. (1990) used stem segments of *A. vera* on MS medium with different hormones and successfully regenerated a large number of plantlets via callus. The best results were obtained on the medium with zeatin 2 ppm + 0.5 ppm NAA. Roy and Sarkar (1991) reported that MS basal medium supplemented with 1 mgL^{-1} 2,4-D and 0.2 mgL^{-1} kinetin gave the best callus induction. *A. vera* has a great future for tissue engineering applications, because it is a unique plant and appealing for physicochemical and biological properties (Shekh et al., 2017).

The shoot tips were used as explants, cultured on different combinations of auxins and cytokinins and were examined. Among all the combinations, no response was observed in MS basal medium with 2, 4-D (0.5 mgL^{-1}) and kinetin (0.2 mgL^{-1}). It was also revealed that regenerated plants were morphologically similar to the mother (control) plant and that the method of micropropagation used in this investigation (axillary bud method) does not usually produce some clones. The result of acclimatization showed that 100% of plantlets survived and grew under greenhouse conditions and were morphologically similar to mother plants. The leaves also started thickening in shade house.

The tissue culture plants were hardened and these plants could face ambient environmental conditions (Bhojwani and Razdan, 1992). Rooted plantlets were transferred from culture bottles to plastic pots containing 1:1 ratio of soil: rice husk. Natali et al. (1990) suggested the mixture of soil, sand and perlite. While Aggarwal and Barna (2004) suggested mixture of soil and farmyard manure, and Hashemabadi and Kaviani (2008) suggested the mixture of cocopeat and perlite. There is decrease in the glycoprotein, verectin (Yagi et al., 2000) and barbaloin content in the clonally regenerated plants of *A. vera*. According to Yagi et al. (2006), the clonally induced mutations are associated with the phenotypic variation

Figure 5. (a-c) Hardened plantlets of twelve accessions transferred to soil in bigger pots: (1) IC111267, (2) IC111269, (3) IC111271, (4) IC111272, (5) IC111279, (6) IC111280, (7) IC471882, (8) IC471883, (9) IC471884, (10) IC471885, (11) *Aloe* CIM-Sheetal (CAL14), (12) wild *Aloe vera* (local).

observed in *A. vera*. The present results show the occurrence of variation in morphological and biochemical characteristics when compared with the mother plants and their tissue culture generated plants. The extent of variation, however, differs from accession to accession. This could be related to differences in the genotypes of various accessions.

Hardening of plantlets

After 30 days of culture on rooting media, the plantlets were successfully acclimatized. The pots (8×6 cm) were kept and readily filled with garden soil, compost and sand in the proportion of 2:1:1, respectively. The plants were then transplanted into the pots, then thoroughly watered and kept under plastic house having 80% humidity and 31°C temperature for ten days. Then the plants were shifted to shade house with less humidity and indirect sunlight. After 15 days, the hardened plantlets were transferred to the soil. The result of acclimatization showed that 100% of plantlets survived to grow under greenhouse conditions and were morphologically similar to mother plants (Figure 5a to c). The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary. The overall procedure of micro propagation flow diagram is as shown in Figure 6. The tissue culture showed the rapid production of large number of true type plants. Somaclonal variations are therefore undesirable. However, in the present case, the somaclonal variants can be a valuable source of genetic variation in the germplasm. The somaclonal variants thus obtained need detailed genetic characterization before being put to use. Genetic variation is an essential component of any conventional breeding programme. Aloe germplasm lacks natural genetic recombination mechanism due to the absence of sexual reproduction. The tissue culture technology has great potential for induction of genetic variability.

Conclusions

The plant of *A. vera* shows slight morphological differences in stems and leaf length, but as they had been collected from different areas which have different environmental conditions, these changes are exceptional since all the accessions collected from different places were cytologically diploid with chromosome number 2n=14 and maintained in the same environmental conditions at Indian Immunologicals Ltd, Hyderabad. It was observed that variability can be related to genetic makeup/genetic diversity. Each explant gave rise to 8.84 shoots in 7 weeks and total number of 15 to 35 shoots/cultures was produced from single explants. Formation of the roots was observed best in *Aloe* CIM-Sheetal and IC111271 within four weeks after inoculation of rooting. The concentration of MS media played a significant role in shoot initiation, proliferation, rooting and regeneration of plant; this depends on the genotypic variation of the explants and is reinforced by the suitable cultural and environmental conditions. This study standardized the surface sterilization procedure and less expensive media composition with high-frequency regeneration as well as for conservation of an important miracle medicinal plant of *A. vera*. So, this protocol has been proven to potentially save the high yielding species from extinction and facilitates germplasm conservation, considering its safer sterilization method and efficient plant *in vitro* propagation system. It would be interesting to collect *A. vera* accessions from different regions and compare the morphological as well as tissue culture results together and this study will be useful for conservation of endangered *Aloe* spp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

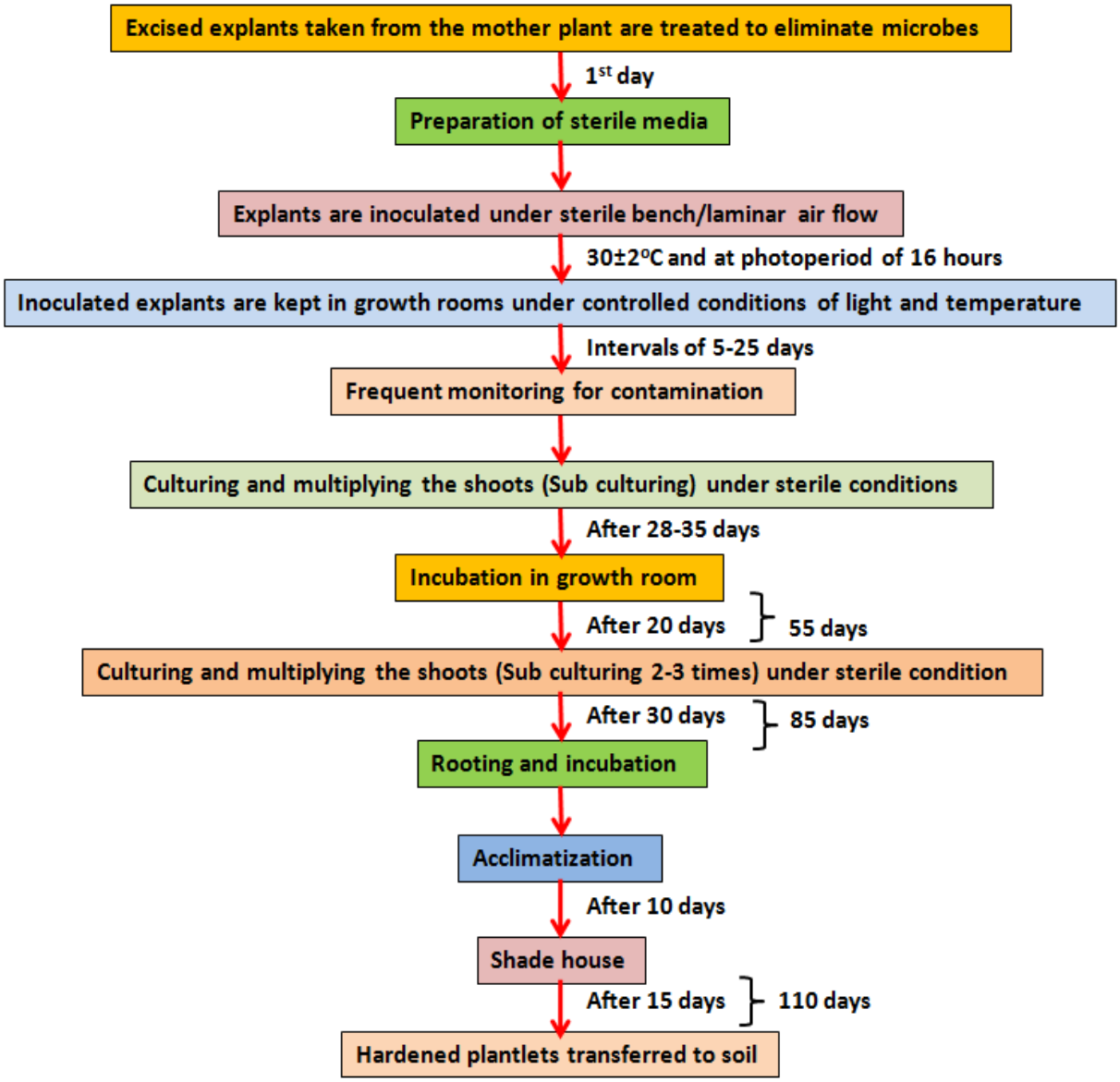

Figure 6. Flow diagram showing the overall procedure of *in vitro* culture of *Aloe barbadensis* Mill.

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