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African Journal of Biotechnology

Full Length Research Paper

Efficient plant regeneration protocol for finger millet [*Eleusine coracana* (L.) Gaertn.] via somatic embryogenesis

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In the present study, an efficient protocol for somatic embryogenesis and plant regeneration was established in six finger millet varieties (GBK-043137, GBK-043128, GBK-043124, GBK-043122, GBK-043094 and GBK-043050). Shoot tips from 3 days *in vitro* grown plants were inoculated on MS supplemented with various concentrations and combinations of α-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP) and kinetin for callus induction and somatic embryogenesis. For shoot regeneration, somatic embryos were cultured on various concentrations of BAP, while root induction was done using different concentrations and combinations of NAA, kinetin, BAP and 2,4-D. Acclimatization of regenerated plants was tested using forest soil, cocopeat, manure, sand and fertilizer either singly or in combination. Best callus formation was achieved on 2.5 mg/l of 2,4-D and 1.5 mg/l BAP with a mean of 12.33±0.33 on variety GBK-043128 while shooting and rooting were best on 1.75 mg/l BAP with a mean of 25.07±0.64 and 1.0 BAP+0.25 NAA with a mean of 15.00±2.2, respectively. Best acclimatization was attained using soil, sand and fertilizer on GBK-043094. Plants regenerated were morphologically similar to *in vivo* plants with 97% survival rate. Moreover, they were fertile and able to set viable seeds. This efficient protocol has the potential for crop improvement and genomic studies.

Key words: Finger millet, plant regeneration, shoot tips, somatic embryogenesis.

INTRODUCTION

Finger millet [*Eleusine coracana* (L.) Gaertn.], which is a small cereal crop that is indigenous to East Africa, is cultivated in arid and semi-arid areas of eastern Africa and south Asia (Sood et al., 2016). It is tetraploid ($2n = 4x = 36$) and belongs to the Poaceae family and Chloridoideae sub-family (Gimode et al., 2016). Finger millet is a highly nutritious cereal high in proteins, vitamins and minerals. Finger millet is one of the potential

grain crops for food and nutritional security, climate resilient farming and agricultural diversification. Despite its significance as a subsistence crop, little attention has focused on the crop's improvement programs, probably since it is considered with minimal monetary significance in contrast to maize, wheat and rice. Finger millet production and yield is constrained by lack of improved varieties, weeds, diseases and pests, limited uses, unpredictable markets, limited research and moisture stress in dry areas (Oduori, 2005). Varieties with new traits emerging as one will help the plant cope with adverse challenges associated with biotic and abiotic stress. The world's increasing demand for finger millet offers a golden opportunity to develop efficient, quick and reproducible strategies and techniques for important finger millet local varieties addressing complex traits such as grain quality, biotic and abiotic stress resiliencies.

Over the years, conventional breeding has been used for traits improvement in finger millet with some success. However, conventional plant breeding is tedious, time consuming and mostly dependent on environment (Miah et al., 2013). As an alternative, biotechnological techniques such as genetic engineering and genome editing techniques, which relies on the availability of *in vitro* plant regeneration systems, provides a powerful tool for genetic manipulation of finger millet. Genetic engineering of finger millet for improved varieties is stymied because of absence of a proficient plant tissue culture strategy with high regeneration frequency. To the authors' knowledge, there is no report on tissue culture and transformation for African finger millet cultivars. Just like other cereals, finger millet regeneration can be accomplished through somatic embryogenesis and organogenesis. Somatic embryogenesis of finger millet is one of the most preferred approach due to production of large numbers of plantlets and its application for genetic transformation technology. Several factors such as the explant source, their developmental stage and plant growth regulators affect this morphogenetic route (Sudhakar et al., 2004). Finger millet has been considered a recalcitrant crop to tissue culture and genetic transformation. Only limited reports are available to date on regeneration and genetic transformation of finger millet finger millet varieties particularly of African origin (Gupta et al., 2017). In these reports, the low rate of embryo initiation, maturation, germination and development into plantlets often remain a major challenge. The present work reports a robust and reliable procedure for the establishment of an efficient and reproducible regeneration through system somatic embryogenesis of finger millet using shoot tips that will be useful for the genetic improvement of this crop.

MATERIALS AND METHODS

Plant material and explant preparation

Seeds of finger millet: GBK-043137, GBK-043128, GBK-043124, GBK-043122, GBK-043094 and GBK-043050 were obtained from Kenya Agricultural and Livestock Research Organization gene bank, at Muguga, Kenya. The seeds were soaked for 30 min in sterile distilled water to dehusk them, followed by surface sterilization with 70% ethanol and 20% sodium hypochlorite containing a few drops of Tween 20 for 20 min. Surface sterilized seed were rinsed three times with sterile distilled water and germinated aseptically on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), supplemented with 3% sucrose, 0.3% gelrite and pH 5.8. The culture bottles were incubated at 25±2°C in the dark for germination for three days.

Callus induction and somatic embryo development

Shoot tips (4 to 6 mm) from *in vitro* germinated plants were aseptically excised and cultured on callus induction medium (CIM) for six weeks in the dark. The CIM comprised of MS supplemented with 30% sucrose and various plant growth hormones (PGRs) either singly or in combinations comprising of NAA (2.0, 2.5, 3.0 and 4.0 mg/l); 2,4-D + BAP (1.5 + 0.5, 2.0 + 1.0, 2.5 + 1.5, 3.0 + 1.5 mg/l); 2,4-D + KN (1.5 + 0.5, 2.0 + 0.5, 2.5 + 1.0, 3.0 + 1.0 mg/l) and NAA + KN (1.5 + 0.5, 2.0 + 0.5, 2.5 + 1.0, 3.0 + 1.0 mg/l). Explants cultured on MS media without plant PGRs were taken as control. Cultures were incubated at 25±2°C in the dark for about four weeks to promote the formation of callus. The explants were subcultured to a fresh media after every two weeks.

Calli that formed were transferred onto embryo induction medium (EIM) containing MS basal salts supplemented with 30 g/l sucrose and 1.75 mg/l of BAP and incubated at 25±2°C in dark for 8 weeks until differentiation into embryo-like structures were observed. The calli were subcultured onto fresh EIM medium after every two weeks.

Shoot proliferation and root formation

Mature embryos were cultured on shoot induction medium (SIM) comprising of MS supplemented with 30% sucrose and different concentrations of BAP (1, 1.5, 1.75 and 2 mg/L). The SIM was also used for elongation with subculture every two weeks up to four subcultures. The number of shoots formed were counted every four weeks and recorded. Shoots formed were cultured on root induction media (RIM) comprising of MS medium supplemented with 30% sucrose and 1.0 KN+0.25 NAA, 1.0 BAP + 0.25 NAA, 1.0 KN + 0.25 2,4-D and 1.0 BAP + 0.25 2,4-D for root formation and development for four weeks. The developed roots were observed and recorded.

Hardening and acclimatization

Rooted plantlets were rinsed with sterile distilled water to remove the excess media. The plantlets were transferred to the greenhouse for acclimatization on four media regimes: forest soil only, cocopeat, forest soil + sand + manure (2:1:1) and forest soil + sand + fertilizer (4:2:0.05). The plants were watered on regular intervals. Data on

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Table 1. Germination efficiencies of six varieties of finger millet.

Variety	Number of seeds	Germination efficiency
GBK-043137	480	74.50±2.06[a]
GBK-043128	500	65.50±2.05[a]
GBK-043124	514	62.50±3.13[a]
GBK-043122	670	40.33±1.45[b]
GBK-043094	510	65.33±4.03[a]
GBK-043050	460	71.00±3.64[a]

number of leaves, plant height and colour was collected after 2, 4 and on the sixth week from the day of hardening off. Survival of plantlets was recorded after 3 weeks [Survival plantlet (%) = (surviving plantlets/total plantlets) x 100).

Statistical analysis

All data were expressed as mean ± standard. The data were analysed using ANOVA with Minitab statistical computer software v.17. Means were separated using the Fisher's protected LSD test at a confidence level of 95% ($p \leq 0.05$).

RESULTS

Effects of plant growth regulators on callus induction

It was possible to geminate seeds of all the selected varieties *in vitro* using the MS hormone free medium, albeit with different gemination efficiencies. GBK-043137 exhibited the highest gemination efficiency of 75% followed by GBK-043050, GBK-043128, GBK-043094 and GBK-043124 with seed gemination efficiencies of 71, 66, 65 and 63%, respectively. Variety GBK-043122 had the least seed germination efficiencies of 40% (Table 1). There was significant difference between GBK-043137, GBK-043050, GBK-043128, GBK-043094 and GBK-043124, and GBK-043122 in terms of seed germination efficiencies on MS hormone free medium (Table 1) ($p<0.05$). However, for varieties GBK-043137, GBK-043050, GBK-043128, GBK-043094 and GBK-043124, there was no significant difference, in terms of seed germination efficiencies (Table 1) ($p>0.05$). All geminated plants exhibited normal phenotype (Figure 1a).

It was possible to initiate calli in all the finger millet varieties at all the different concentrations and combinations of NAA, 2,4-D, BAP and kinetin, however with different callus formation frequencies. Different combinations and concentrations of NAA (2.0, 2.5, 3.0 and 4.0 mg/l); 2,4-D + BAP (1.5 + 0.5, 2.0 + 1.0, 2.5 + 1.5, 3.0 + 1.5 mg/l); 2,4-D + kinetin (1.5 + 0.5, 2.0 + 0.5, 2.5 + 1.0, 3.0 + 1.0 mg/l), NAA + kinetin (1.5 + 0.5, 2.0 + 0.5, 2.5 + 1.0, 3.0 + 1.0 mg/l) and control were tested for their ability to induce calli and determine best combination and concentration for calli induction of the finger millet varieties. All the treatments produced calli. The callus induction mean percentage ranged from 4.33±0.33 to 12.33±0.33 out of 60 explants based on the type and concentration of PGRs in the medium (Table 2). The highest callus formation was observed on medium supplemented with 2.5 mg/l 2,4-D + 1.5 mg/l BAP at 12.33±0.33 for variety GBK-043128 (Table 2a). This combination and concentration also had the best response for callus induction on all other varieties when compared with the others. GBK-043094 was the best responding variety with regards to callus induction in all media combinations and concentrations. Medium supplemented with NAA was the worst performing media with 4.33±0.33 callus formation. This medium formed callus like structures at the tip of explant and developed shoots within three days which were fast growing. These explants were subcultured; they turned brown after a week and eventually died off. The controls did not form any callus (Table 2a and b). Explants formed calli that were friable, soft, watery, white in colour, they developed gradually, slowly and were non-regenerative with unorganized morphology, destitute of nodular structures (Figure 1b). Following a month of culture, the greater part of the calli turned out to be light yellow in colour and embryogenic in nature with nodular development (Figure 1c). Explants did not show any change turned brown and eventually black and they died within three weeks of culture.

Regeneration of plants from callus

The rapidly growing friable calluses were subcultured onto MS medium supplemented with 1.75 mg/l of BAP for somatic embryo induction. Somatic embryogenic structures started to form after two weeks culture onto the EIM. The greenish compact and moderate developing calli that developed organized structures and inevitably displayed tissue differentiation were regarded as embryogenic (Figure 1d), as opposed to the non-embryogenic calli that were white or cream, friable and quickly developing (Figure 1e). Some calli of the finger millet varieties turned green and formed globular, heart, torpedo and cotyledonary stages of embryos which appeared progressively after 4 weeks on EIM medium. There was 100% transition from callus to somatic

Figure 1. (a) Three day seedling geminated on the dark; **(b)** Callus induction on shoot tip; **(c)** Embryogenic callus; **(d)** Embryo development; **(e)** Embryo maturation; **(f)** Shoot multiplication; **(g)** Root plants; **(h)** 1 day plantlet undergoing acclimatization on cocopeat media; **(i)** Two weeks after hardening off on cocopeat media.

embryos for all finger millet varieties. After four weeks on EIM in light, the embryo maturation was observed with formation of small shoots (Figure 1f).

Shoot elongation and multiplication

The highest shoot formation frequency was achieved using MS supplemented with 1.75 mg/l on GBK-043094 and GBK-043137 both with a mean of 25.07±0.64 and 25.33±2.95, respectively (Table 3). This concentration of MS and 1.75 mg/l was also highly significant ($P \leq 0.05$) for GBK-043128, GBK-043124, GBK-043050, GBK-043094 and GBK-043137 with mean number of shoots at 23.93±0.74, 22.53±1.03, 23.93±0.74, 25.07±0.64 and 25.33±2.95 respectively, however a lower concentration of 1.5 mg/l was highly significant for GBK-043122. The lowest performing shooting media was 1.0 mg/l on GBK-043050 and 2.0 mg/l on GBK-043050 with a mean number of shoots of 9.80±1.33 and 9.80±2.40 respectively. The height of plants varied from 3 to 6 cm.

Root induction

Shoots (2 to 3 cm in height) cultured on MS media

Table 2a. Callus induction on six varieties of finger miller on different plant growth regulators

Variety	NAA (Mg/L)				2,4-D (Mg/L) + BAP (Mg/L)			
	2.0	2.5	3.0	4.0	1.5 +0.5	2.0 +1.0	2.5 +1.5	3.0 +1.5
GBK-043050	5.00 ± 0.58^{b}	5.67 ± 0.88^{a}	8.67 ± 0.66^{a}	7.33 ± 0.66^{a}	9.66 ± 0.33^{ab}	12.00 ± 1.00^{a}	11.67 ± 0.33^{ab}	11.33 ± 0.33^{a}
GBK-043094	6.67 ± 1.33^{a}	6.00 ± 0.58^{a}	8.33 ± 0.88^{a}	7.00 ± 0.58^{ab}	10.00 ± 0.58^{a}	11.33 ± 0.66^{a}	11.33 ± 0.33^{abc}	10.00 ± 0.55^{ab}
GBK-043122	4.66 ± 0.33^{b}	5.33 ± 1.2^{a}	6.00 ± 0.57^{b}	6.00 ± 0.57^{ab}	8.67 ± 0.33^{bc}	9.66 ± 0.88^{b}	10.30 ± 0.88^{bc}	9.33 ± 0.88^{b}
GBK-043124	5.00 ± 1.00^{b}	6.33 ± 0.33^{a}	8.67 ± 0.67^{a}	7.33 ± 0.66^{a}	9.67 ± 0.33^{ab}	11.66 ± 0.33^{a}	10.00 ± 0.57^{bc}	9.67 ± 0.33^{b}
GBK-043128	5.33 ± 0.33^{ab}	5.67 ± 0.33^{a}	6.00 ± 0.58^{b}	5.66 ± 0.88^{b}	8.33 ± 0.66^{c}	9.67 ± 0.88^{b}	12.33 ± 0.33^{a}	9.33 ± 0.88^{b}
GBK-043137	4.33 ± 0.33^{b}	5.67 ± 0.33^{a}	6.33 ± 0.66^{b}	6.00 ± 0.58^{ab}	8.33 ± 0.67^{c}	9.66 ± 0.86^{b}	10.33 ± 0.88^{c}	9.33 ± 0.88^{b}
Control	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}

Table 2b. Callus induction on six varieties of finger miller on different plant growth regulators.

Variety	2,4-D (Mg/L) + KN (Mg/L)				NAA (Mg/L) + KN (Mg/L)			
	1.5 +0.5	2.0 +0.5	2.5 +1.0	3.0 +1.0	1.5 +0.5	2.0 +0.5	2.5 +1.0	3.0 +1.0
GBK-043050	11.33 ± 0.88^{a}	8.00 ± 0.58^{a}	8.00 ± 1.00^{a}	8.00 ± 0.00^{a}	7.67 ± 1.20^{ab}	6.33 ± 0.88^{a}	6.00 ± 1.15^{a}	5.67 ± 0.88^{b}
GBK-043094	6.67 ± 0.88^{b}	7.33 ± 1.2^{a}	8.00 ± 1.20^{a}	8.00 ± 1.00^{a}	6.00 ± 1.15^{b}	6.00 ± 1.15^{a}	7.67 ± 1.20^{a}	7.00 ± 0.58^{a}
GBK-043122	7.33 ± 0.88^{b}	7.66 ± 0.33^{a}	7.00 ± 1.00^{a}	7.00 ± 1.00^{a}	7.33 ± 0.33^{ab}	7.66 ± 0.88^{a}	6.67 ± 1.20^{a}	6.67 ± 0.33^{ab}
GBK-043124	7.33 ± 1.45^{b}	7.66 ± 0.88^{a}	7.66 ± 0.86^{a}	7.33 ± 1.45^{a}	7.66 ± 0.33^{ab}	6.67 ± 0.88^{a}	7.33 ± 0.88^{a}	7.66 ± 0.33^{a}
GBK-043128	6.67 ± 0.66^{b}	7.33 ± 0.33^{a}	8.33 ± 0.33^{a}	8.33 ± 0.33^{a}	6.33 ± 0.88^{b}	7.00 ± 0.58^{a}	7.33 ± 0.33^{a}	5.66 ± 0.33^{b}
GBK-043137	6.66 ± 0.67^{b}	7.33 ± 0.78^{a}	7.00 ± 1.00^{a}	6.66 ± 0.33^{a}	8.33 ± 1.20^{a}	7.00 ± 1.20^{a}	7.00 ± 1.00^{a}	6.66 ± 0.33^{ab}
Control	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}

Means (± SE) followed by different alphabets in each column are significantly different ($P \leq 0.05$) using Fishers LSD.

Table 3. Shoot induction on BAP.

Variety	BAP (Mg/L)			
	1	1.5	1.75	2
GBK-043050	10.53 ± 1.31^{b}	12.13 ± 0.26^{b}	23.93 ± 0.74^{a}	9.80 ± 2.40^{a}
GBK-043094	19.33 ± 2.75^{a}	12.6 ± 0.72^{b}	25.07 ± 0.64^{a}	9.8 ± 1.33^{a}
GBK-043122	10.40 ± 0.83^{b}	17.33 ± 0.77^{a}	12.60 ± 0.70^{b}	11.93 ± 1.54^{a}
GBK-043124	16.40 ± 1.74^{a}	12.53 ± 1.73^{b}	22.53 ± 1.03^{a}	10.40 ± 0.83^{a}
GBK-043128	9.80 ± 1.33^{b}	12.30 ± 0.41^{b}	23.93 ± 0.74^{a}	10.47 ± 1.85^{a}
GBK-043137	15.60 ± 2.55^{ab}	13.53 ± 1.17^{b}	25.33 ± 2.95^{a}	13.67 ± 1.83^{a}

Means (± SE) followed by different alphabets in each column are significantly different ($P \leq 0.05$) using Fishers LSD.

containing 1.0 KN + 0.25 NAA, 1.0 BAP + 0.25 NAA, 1.0 KN + 0.25 2,4-D and 1.0 BAP + 0.25 2,4-D showed root induction in all six finger millet varieties, after 4 weeks of incubation. The roots started forming within two weeks and proper root system was achieved in four weeks (Figure 1g) with high significant differences observed using 1.0 BAP + 0.25 NAA for varieties GBK-043128, GBK-043124, GBK-043094 and GBK-043050 with mean of 14.60±2.23, 15.00±2.2, 15.00±2.2 and 13.93±2.03, respectively. MS supplemented with 1.0 KN + 0.25 NAA was significantly higher for GBK-043137 and GBK-043122 with a mean of 10.87 ± 0.75 and 11.07 ± 0.93 respectively (Table 4). The best rooting hormone combinations not only had highest rooting but also 100% of the plants formed roots as compared to rest of the combinations.

Hardening and acclimatization

Well-developed plantlets (5 to 6 cm in height) with more than two true leaves from all the regenerated varieties

Table 4. Root induction using various PGRs.

Variety	PGRs			
	1.0 KN+0.25 NAA	**1.0 BAP+0.25 NAA**	**1.0 KN+0.25 2,4-D**	**1.0 BAP +0.25 2,4-D**
GBK-043050	11.60±0.61[b]	13.93±2.03[a]	10.93±0.52[a]	9.27±0.71[a]
GBK-043094	13.67±0.24[a]	15.00±2.20[a]	9.53±0.47[ab]	8.20±0.31[ab]
GBK-043122	11.07±0.93[b]	8.00±0.50[b]	8.3±0.82[bc]	7.20±0.64[b]
GBK-043124	11.67±0.41[b]	15.00±2.2[a]	7.8±0.40[c]	7.20±0.64[b]
GBK-043128	11.87±0.27[ab]	14.60±2.32[a]	10.93±0.52[a]	9.87±0.46[a]
GBK-043137	10.87±0.75[b]	7.50±0.82[b]	7.4±0.31[c]	7.2±0.46[b]

Means (± SE) followed by different alphabets in each column were significantly different (P ≤0.05) using Fishers LSD.

were hardened successfully in pots using four media regimes: forest soil only, cocopeat, forest soil + sand + manure in a ratio and forest soil + sand + fertilizer in 2:1:1 and 4:2:0.05 ratios with 97% survival rate (Figure 1h to i). The number of leaves for all the *in vitro* regenerated finger millet varieties varied between 6.43±1.30 and 11.92±0.96. The number of leaves for all the *in vitro* regenerated finger millet varieties was statistically, not significantly different at two and four six weeks for soil while all the rest were different (Table 5) across the media regimes. Following seven days of incubation in a growth chamber, all the pots were moved to regular environmental conditions with a 100% survival rate with a 100% survival rate. The somatic embryo-derived plantlets grew well and exhibited phenotypic homogeneity as compared to seed-derived field-grown finger millet plants (Figure 1g and h).

The height of the plants at two weeks varied from 3 to 6 cm. The shortest plants at this period were those hardened on soil, sand and manure which were also significantly different on GBK-043124 (Table 6). At four weeks, the tallest plants were produced using cocopeat which was not significantly different across varieties (Figure 1b). The media with the tallest plants at six weeks was soil, sand and manure (52.33±5.56) on variety GBK-043128 which was significantly different while soil + sand + fertilizer produced the shortest plants (Table 6).

DISCUSSION

In this study, shoot apical meristems derived from mature seeds of several finger millet varieties germinated in culture used for the successful induction of embryogenic calli and subsequent plant regeneration. Although, six Kenyan farmers preferred finger millet varieties were used, this regeneration system can be extended into a range of agronomically important African finger millet varieties. Explants derived from mature seeds are considered an excellent source material for biotechnological application due to easy storage and accessibility to large amounts of uniform quality explant material (Sudhakar et al., 2004). Shoot apical meristems have been used successfully in the regeneration systems in cereals as starting material to obtain stable transformation in barley, wheat, maize, sorghum and millet (Sticklena and Orabya, 2005). The use of shoot apex in regeneration is critical since it can divide to produce viable new organs such as leaves, stems and adventitious roots (Itoh et al., 2006). Shoot apex is also most beneficial for its quick development therefore it can allow rapid development of plants (Ceasar and Ignacimuthu, 2008; Dey et al., 2012). Auxins have been shown to play an important role in inducing callus (Anjaneyulu et al., 2011). Therefore, in the current study, it was used alone or in combination with cytokinins. It was observed that by increasing levels of NAA across varieties resulted in the formation of callus-like structures at the base of the tip of explant then it started elongating and eventually shoots were formed which was higher as compared to other plant growth regulators. This trend is consistent with the works of Ceasar and Ignacimuthu (2008) who observed that after 5 weeks, NAA induced callus remained non-responsive. The previous reports on cereals tissue culture dealt with different auxins and cytokinins at different concentrations also showed the superiority of 2,4-D over the other auxins. The presence of NAA growth regulator was also found to be inhibitory for plant regeneration as well as callus proliferation. Medium supplemented with NAA was the worst performing media with 4.33±0.33 to 10.00±0.58 callus formation (Table 2a). It is therefore not understood why NAA performed poorly on the finger millet varieties tested. More studies should be done to investigate this callus proliferation as well as plant regeneration inhibitory performance.

Plant growth regulators auxins alone and cytokinins or in combination plays a very important role inducing callus and its proliferation (Thomas and Maseena, 2006). The degree of embryonic callus was improved by a combination of 2,4-D and BAP at 2.5 mg/l 2,4-D +0.5 mg/l of BAP for GBK-043137, GBK-043128, GBK-043122 and GBK-043050. A higher level of 3.0 mg/L 2,4-D + 1.5 mg/L was significant for GBK-043124 and GBK-043094. This is in agreement with the works of Anjaneyulu et al. (2011) who used 2 mg/l ,4-D + 1.0 mg/l of BAP obtaining

Table 5. Mean number of leaves at two, four and six weeks hardened using soil, cocopeat, soil, sand and manure, and soil, sand and fertilizer at the greenhouse.

Variety	2 weeks				4 weeks				6 weeks			
	SSF	Soil	Cocopeat	SSM	SSF	Soil	Cocopeat	SSM	SSF	Soil	Cocopeat	SSM
GBK-043050	10.75 ± 0.90^{abc}	8.08 ± 0.96^{a}	7.17 ± 0.30^{b}	9.25 ± 0.58^{a}	8.08 ± 0.22^{c}	7.67 ± 1.08^{a}	8.50 ± 0.63^{b}	8.50 ± 0.66^{a}	8.17 ± 1.04^{b}	7.92 ± 1.40^{abc}	11.00 ± 2.10^{a}	11.00 ± 2.27^{a}
GBK-043094	9.42 ± 0.44^{bc}	8.83 ± 0.30^{a}	7.58 ± 0.51^{b}	9.00 ± 0.88^{a}	11.41 ± 0.22^{a}	10.00 ± 0.38^{a}	8.83 ± 0.58^{ab}	11.33 ± 0.51^{a}	11.75 ± 0.87^{a}	9.92 ± 1.37^{ab}	11.50 ± 1.51^{a}	11.00 ± 1.18^{a}
GBK-043122	11.00 ± 0.38^{ab}	9.17 ± 1.26^{a}	7.92 ± 0.60^{b}	9.08 ± 0.44^{a}	10.00 ± 0.95^{ab}	9.33 ± 1.16^{a}	10.17 ± 0.79^{ab}	9.67 ± 0.42^{a}	8.00 ± 0.43^{b}	7.67 ± 0.68^{bc}	10.50 ± 0.76^{a}	10.67 ± 1.23^{a}
GBK-043124	11.42 ± 0.30^{a}	9.25 ± 1.39^{a}	8.58 ± 0.51^{ab}	8.58 ± 1.10^{a}	9.25 ± 0.58^{bc}	9.50 ± 1.53^{a}	10.33 ± 0.58^{ab}	9.50 ± 1.04^{a}	6.43 ± 1.30^{b}	7.00 ± 0.66^{c}	10.50 ± 0.58^{a}	11.00 ± 1.18^{a}
GBK-043128	8.83 ± 0.46^{c}	9.42 ± 0.98^{a}	7.08 ± 0.30^{b}	8.17 ± 1.17^{a}	9.00 ± 0.80^{bc}	10.08 ± 7.26^{a}	10.17 ± 0.96^{ab}	9.08 ± 1.40^{a}	6.50 ± 0.58^{b}	9.58 ± 0.46^{abc}	10.58 ± 1.16^{a}	11.25 ± 1.50^{a}
GBK-043137	11.92 ± 0.96^{a}	10.42 ± 0.98^{a}	10.00 ± 0.87^{a}	9.67 ± 1.45^{a}	8.25 ± 1.44^{bc}	10.42 ± 0.30^{a}	10.83 ± 0.79^{b}	9.00 ± 1.38^{a}	8.00 ± 0.66^{b}	10.58 ± 0.58^{a}	9.50 ± 0.86^{a}	10.58 ± 2.02^{a}

Table 6. Mean height of plants at two, four and six weeks hardened using soil, cocopeat, soil, sand and manure, and soil, sand and fertilizer at the greenhouse.

Variety	2 weeks				4 weeks				6 weeks			
	SSF	Soil	Cocopeat	SSM	SSF	Soil	Cocopeat	SSM	SSF	Soil	Cocopeat	SSM
GBK-043050	3.75 ± 0.52^{bc}	3.63 ± 0.13^{c}	3.88 ± 0.26^{a}	4.58 ± 0.51^{a}	5.16 ± 0.65^{b}	5.33 ± 0.16^{c}	7.54 ± 0.52^{b}	8.33 ± 0.96^{b}	6.00 ± 0.25^{b}	14.75 ± 1.89^{c}	26.25 ± 4.59^{cd}	37.67 ± 5.41^{ab}
GBK-043094	6.33 ± 0.22^{a}	4.00 ± 0.14^{bc}	4.42 ± 0.79^{a}	4.33 ± 0.08^{a}	8.67 ± 0.42^{a}	6.00 ± 0.29^{bc}	8.17 ± 0.65^{ab}	7.92 ± 0.55^{b}	12.33 ± 1.02^{a}	9.58 ± 0.96^{c}	21.67 ± 3.35^{d}	31.33 ± 3.97^{b}
GBK-043122	5.17 ± 0.46^{ab}	4.75 ± 0.38^{ab}	5.50 ± 0.80^{a}	4.58 ± 0.36^{a}	5.33 ± 0.65^{b}	7.08 ± 0.58^{b}	10.92 ± 0.54^{ab}	8.75 ± 0.52^{b}	7.17 ± 1.34^{b}	6.58 ± 4.41^{b}	44.25 ± 5.86^{ab}	34.42 ± 4.29^{b}
GBK-043124	5.08 ± 0.71^{ab}	4.75 ± 0.50^{ab}	4.33 ± 1.21^{a}	2.79 ± 0.27^{b}	4.75 ± 0.80^{b}	6.75 ± 0.50^{b}	9.50 ± 1.63^{ab}	6.88 ± 0.62^{b}	6.81 ± 0.79^{b}	28.33 ± 3.08^{ab}	39.58 ± 4.03^{abc}	36.92 ± 5.93^{b}
GBK-043128	6.33 ± 0.63^{a}	5.58 ± 0.08^{a}	5.50 ± 0.80^{a}	5.38 ± 0.62^{a}	8.08 ± 0.60^{a}	9.58 ± 0.08^{a}	11.25 ± 0.76^{a}	11.17 ± 0.33^{a}	12.33 ± 1.02^{a}	35.83 ± 2.26^{a}	46.92 ± 6.80^{a}	52.33 ± 5.56^{a}
GBK-043137	3.42 ± 0.71^{c}	4.58 ± 0.08^{b}	3.50 ± 1.15^{a}	5.29 ± 0.34^{a}	5.92 ± 0.74^{b}	10.42 ± 0.44^{a}	10.17 ± 1.80^{ab}	12.33 ± 0.55^{a}	6.58 ± 0.44^{b}	27.67 ± 1.56^{b}	30.75 ± 1.73^{bcd}	40.42 ± 4.56^{ab}

highest callus formation. Growth regulators at varying concentrations cause variability in plantlet regeneration. The callus obtained on both varieties across the hormones at earlier stages was white and compact but as it approached about six weeks, some were yellow and brown watery and soft. This agrees with the works of Yemets et al. (2013) who obtained three types: (1) callus white, compact, well-structured callus; (2) yellow or lemon, less compact, globular callus; (3) yellow, watery, soft callus. They were able to identify that the first type of callus was superior and was produced only when NAA was added to the 2,4-D + KIN combinations. This research confirms that the superior callus can also be obtained by combination of 2,4-D and BAP which is variety independent. In all the cases, use of MS alone which was used as control turned brown then died off without producing any callus.

Two step embryo maturation containing cytokinin has proven to be effective for plantlet regeneration (Xie and Hong, 2001; Seabrook and Douglass, 2001). This was done using BAP and it was found out that the optimum for both varieties was at 1.75 mg/l. This is similar to the works of Patil et al. (2009) who found out that the best performing concentration was 2.0 mg/l and higher concentrations than this had negative effect on shoot induction. A combination of both cytokinins and auxins was used for root regeneration and it was observed that 1.0 KN + 0.25 NAA was significantly higher as compared to the rest of treatments for GBK-043137; this is in agreement with the works of Anjaneyulu et al. (2011) though the average number was lower in this study for varieties GBK-043094 1.0 BAP+0.25 NAA produced significantly higher shoots and it has the highest number. In both treatments, there was 100% root formation.

Soil, sand and fertilizer regime were initially the best performing at two weeks in terms of leaf number and height because plants are able to acclimatize better but it was overtaken by cocopeat which did not retain water easily and at the first weeks, the plants showed signs of witling. However, with frequent watering, the plants improved significantly and at four weeks, they were the healthiest plants that displayed green

colour and their size of stem was also the largest. The plants were acclimatized and hardened successfully with 97% survival rate. This high survival rates could be due to well-developed root system and the greenhouse conditions. The plants that died (80%) were those hardened on cocopeat; this could be due to the low capacity to retain water as compared to other media. Plants were regenerated after eight weeks of culture.

Conclusion

An effective, simple and reproducible system for somatic embryogenesis and regeneration of Kenyan finger millet varieties was developed using shoot apical meristems. This was achieved by combining auxin and cytokinin at appropriate concentrations and combinations to stimulate somatic embryogenesis from shoot explants and plant regeneration stages. The study has also proved that finger millet is recalcitrant to regeneration; its tissue culture is genotype dependent and also heavily influenced by environmental conditions under which the explants is grown. This reproducible protocol will form the basis for the improvement of this crop by transgenic technologies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

2,4-D, 2,4-Dichlorophenoxyacetic acid; **BAP,** benzylaminopurine; **MS,** Murashige and Skoog medium; **NAA,** α-naphthaleneacetic acid; **CIM,** callus induction media; **EIM,** embryo induction medium; **SIM,** shoot induction medium; **KN,** kinetin; **PGR,** plant growth hormones.

REFERENCES

Anjaneyulu E, Attitalla H, Hemalatha S, Eharath Raj S, Balaji M (2011). An efficient protocol for callus induction and plant regeneration in finger millet (*Eleusine coracana* L.) World Applied Sciences Journal, 12(7):919-923.

Ceasar SA, Ignacimuthu S (2008). Efficient somatic embryogenesis and plant regeneration from shoot apex explants of different Indian genotypes of finger millet (*Eleusine coracana* (L.) Gaertn.). In Vitro Cellular & Developmental Biology-Plant, 44:427-435.

Dey M, Bakshi S, Galiba G, Sahoo L, Panda SK (2012). Development of a genotype independent and transformation amenable regeneration system from shoot apex in rice (*Oryza sativa* spp. indica) using TDZ. 3 Biotechnology, 2:233-240.

Gimode D, Odeny DA, de Villiers EP, Wanyonyi S, Dida MM, Mneney EE, Muchugi A, Machuka J, de Villiers SM 2016). Identification of SNP and SSR markers in finger millet using next generation sequencing technologies. PLoS one, 11(7): 1-21.

Gupta SM, Arora S, Mirza N, Pande A, Lata C, Puranik S, Kumar J, Kumar A (2017). Finger millet: a “certain” crop for an “uncertain” future and a solution to food insecurity and hidden hunger under stressful environments. Frontiers in Plant Science, 8(643):1-14.

Itoh JI, Sato Y, Nagato Y, Matsuoka M (2006). Formation, maintenance and function of the shoot apical meristem in rice. Plant Molecular Biology, 60:827-842.

Miah G, Rafii MY, Ismail MR, Puteh AB, Rahim HA, Asfaliza R, Latif MA (2013). Blast resistance in rice: a review of conventional breeding to molecular approaches. Molecular Biology Reports, 40(3): 2369-2388.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15:473-497.

Oduori COA (2005). The importance and research status of finger millet in Africa. Paper Presented at The McKnight Foundation Collaborative Crop Research Program Workshop on Tef & Finger Millet: Comparative Genomics of the Chloridoid Cereals at the Biosciences for East and Central Africa (BECA) ILRI, Nairobi, Kenya, 28 – 30 June 2005.

Patil SM, Sawardekar SV, Bhave SG, Sawant SS, Jambhale ND, Gokhale NB (2009). Development of somaclones and their genetic diversity analysis through RAPD in finger millet (*Eleusine coracana* L. Gaertn.). Indian Journal of Genetics and Plant Breeding, 69: 132-139.

Seabrook JEA, Douglass LK (2001). Somatic embryogenesis on various potato tissues from a range of genotypes and ploidy levels. Plant Cell Reports, 20:175-182.

Sood S, Kumar A, Babu BK, Gaur VS, Pandey D, Kant L, Pattnayak A (2016). Gene discovery and advances in finger millet [*Eleusine coracana* (L.) Gaertn.] Genomics-An important nutri-cereal of future. Frontiers in Plant Science, 7(1634):1-17.

Sticklena MB, Orabya HF (2005). Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. In Vitro Cellular and Developmental Biology-Plant, 41:187-200.

Sudhakar D, Duc LT, BuiBa-Bong BB, Tinjuangjun P, Maqbool SB, Valdez M, Jefferson R, Christou P (2004). An efficient rice transformation system utilizing mature seed-derived explants and a portable, inexpensive particle bombardment device. Transgenic Research, 1:289-294.

Thomas TD, Maseena EA (2006). Callus induction and plant regeneration in *Cardiospermum halicacabum* (L.) an important medicinal plant. Scientia Horticulturae. 108:332-336.

Veluthambi K, Gupta K, Sharma A (2003). The current status of plant transformation technologies. Current Science, 84: 368-380.

Xie DY, Hong Y (2001). Regeneration of *Acacia mangium* through somatic embryogenesis. Plant Cell Reports, 20:34-40.

Yemets AI, Bayer GA, Blume YB (2013). An effective procedure for *in vitro* culture of *Eleusine coracana* (L.) Gaertn. and its application. ISRN Botany, 21: 503-510.

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Full Length Research Paper

Marine killer yeast *Metschnikowia saccharicola* active against pathogenic yeast in crab and an optimization of the toxin production

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A marine yeast strain, DD21-2 was isolated from sediments in Dandong, China, which has killer activity against yeast *Metschnikowia bicuspidata* WCY (pathogenic to crab, *Portunus trituberculatus*). Routine identification, sequence analysis of 26S rDNA and ITS sequencing showed that the strain was *M. saccharicola* DD21-2, and has not been previously reported as having killer activity against *M. bicuspidata* WCY. To optimize the production of the killer toxin by *M. saccharicola* DD21-2, the interaction effects of fermentation process variables were investigated by Response Surface Methodology (RSM). The following reaction factors were selected in screening experiments: Inoculum concentration (2 to 6%), pH (5.0 to 6.0), temperature (20 to 28°C), and fermentation time (2 to 3 days), and the diameter of the inhibition zone produced by the killer toxin was used as a response variable. A quadratic regression model of killer toxin activity was established by regression analysis and significance testing (P test). The results identified the following parameters as optimal for maximal production of the killer toxin by *M. saccharicola* DD21-2: Fermentation temperature 28°C, pH 5.5, fermentation time 2.7 days, and inoculum concentration 4.1% (v/v). Thus, RSM was effective in determining the best conditions for killer toxin production, suggesting the practical usage of this marine yeast in the investigation of process variables.

Key words: Marine killer yeast, killer toxin, response surface methodology (RSM), fermentation, optimization.

INTRODUCTION

Many studies have shown that some marine yeast species are pathogenic to marine animals (Xu, 2005; Wang et al., 2008; Kaewwichian et al., 2012). A condition called 'milky disease' caused by yeast *Metschnikowia*

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bicuspidata has been detected in crab *Portunus trituberculatus* cultured in 2001 in Zhoushan, Zhejiang Province, China (Xu et al., 2003). However, anti-yeast compounds such as nystatin, benzalkonium bromide, and extracts of gold thread root and garlic are toxic to the crab, so killer toxins produced by some yeast species against pathogenic yeasts may be a good alternative (Philliskink and Young, 1975). Studies have shown that killer yeasts and the killer toxins they produce have antimicrobial activity and can be used to control the growth of pathogenic yeasts in humans, animals and plants (Comitini et al., 2004; Magliani et al., 2008; Chi et al., 2010; Wang et al., 2013).

Yeast-derived killer toxins have been widely used to control harmful yeast growth in industrial fermentation systems, food production, animal breeding, agriculture, and medicine as antifungal drug formulations (Magliani et al., 2008; Wang et al., 2013). In the food industry, killer toxins inhibit the growth of wild yeast and prevent contamination, thus providing a good system environment and improving product quality (Schmitt and Breinig, 2002). In aquaculture, some diseases of marine animals, including *P. trituberculatus* can be controlled by certain marine killer toxin-producing yeast (Wang et al., 2007; Chi et al., 2010).

Optimizing the process of yeast fermentation is important to obtain maximum yield of killer toxins (Bandeira et al., 2006; Çorbacı and Uçar, 2017). However, the reaction system cannot be comprehensively analyzed using a traditional one-variable-at-a-time technique if more than one variable is present (Bezerra et al., 2008). Therefore, it is necessary to employ an appropriate statistical approach to evaluate the relationship among measurable variables. Response surface methodology (RSM) comprises mathematical and statistical techniques aimed at optimizing system response influenced by several independent variables (Bezerra et al., 2008), such as fermentation temperature and the amount of starter culture to obtain the best performance during fermentation (Yaakob et al., 2011). The objective of this study was to screen marine yeast species for the production of killer toxins against pathogenic yeast and determine the optimal conditions for killer toxin production in culture by investigating the interactions between process variables providing a basis for further research of disease prevention and control in marine aquaculture.

MATERIALS AND METHODS

Yeast strains and media

M. bicuspidata WCY (collection number 2E00088 at the Marine Microorganisms Culture Collection of China) was confirmed pathogenic yeast in *P. trituberculatus* (Wang et al., 2007). *Candida tropicalis* and *Candida albicans* isolated from different marine environments were used as susceptible yeast strains. Yeast strains were grown in yeast extract peptone dextrose (YPD) medium containing 1.0% yeast extract, 2.0% glucose, 2.0% peptone, and 3% agar. The medium for killer toxin production was prepared aseptically by adding 2% NaCl and 15% glycerol to YPD, and medium pH was initially adjusted to 4.5 with 0.05 mol/L citric acid-hydrogen phosphate disodium buffer. Killer toxin activity was assayed in YPD agar supplemented with 3 mg/mL methylene blue dissolved in ethanol (final concentration 0.003%) (Guo et al., 2013a).

Screening of marine killer yeast

Each yeast strain from the slants was grown in YPD liquid medium at 28°C, 140 rpm for 24 h; then, 2 ml of culture was centrifuged at 4°C for 5 min (5,000 × g) and washed three times with sterile water. Cell suspension was adjusted to 1×10^7 cells/ml, and 0.2 ml was inoculated into assay YPD agar plates seeded with susceptible pathogenic yeast strains. After 2 to 3 days of incubation at 28°C, a clear killing zone was observed around the colonies of killer yeast, which were then selected based on killing activity (determined as the ratio of the inhibition-zone diameter to colony diameter) (Wang et al., 2007; Peng, 2010).

Yeast identification

Routine yeast identification was conducted as described previously (Kurtzman and Fell, 2011; Kaewwichian et al., 2012). DNA extraction, PCR for the amplification of yeast 26S rDNA, and ITS were performed as previously reported by Chi et al. (2007). Primers for the D1/D2 domain of 26S rDNA were: Forward NL-1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and reverse NL-4 5′-GGTCCGTGTTTCAAGACGG-3′ (Sugita et al., 2003). Primers for ITS1/ITS4 were: Forward ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and reverse ITS4 5′-TCCTCCGCTTATTGATATGC-3′(Guo et al., 2013b). The reaction volume (50 mL) contained 25 mL SuperMix (2 ´ Easy Taq), 0.1 mmol/L NL-1 or NL-4 (1 mL), 0.1 mmol/L ITS1 or ITS4 (1 mL), 50 ng/mL template DNA (2 mL), and H_2O (21 mL). PCR was performed in the Eppendorf Gradient Mastercycler (Shanghai Eternal Medical Instrument Co., Ltd., Shanghai, China) under the following conditions: Initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated via agarose gel electrophoresis and the ITS and D1/D2 26S rDNA fragments were sequenced by Invitrogen Biotechnology (Shanghai, China).

Phylogenetic analysis

Yeast phylogenetic analysis was performed by the neighbor joining method using MEGA 5.1 (Tamura et al., 2011). Evolutionary distances were calculated using the p-distance model of MEGA 5.1, and bootstrap analysis was performed on 1,000 random resamplings. Reference sequences were retrieved from GenBank (accession numbers are indicated on the tree).

Measurement of killer toxin activity

Killer toxin activity was determined by a diffusion test using Oxford cups (6.0 × 10.0 mm) placed on the surface of assay agar plates (Santos et al., 2000) seeded with strain WCY. Killer toxin supernatant (250 µL) was added to each cup, and plates were incubated at 28°C for 2 days; the diameter of the inhibition zone was used as a measure of killer toxin activity (Peng, 2010). And each experiment was repeated 3 times.

Killer toxin production

Before designing the RSM experiment, selecting the inoculum

Table 1. Factors and levels of RSM test.

Factor	Level			
	Symbol	-1	0	+1
Inoculum concentration (%)	X_1	2	4	6
pH value of fermentation broth	X_2	5.0	5.5	6.0
Fermentation time (days)	X_3	2	2.5	3
Fermentation temperature (°C)	X_4	26	28	30

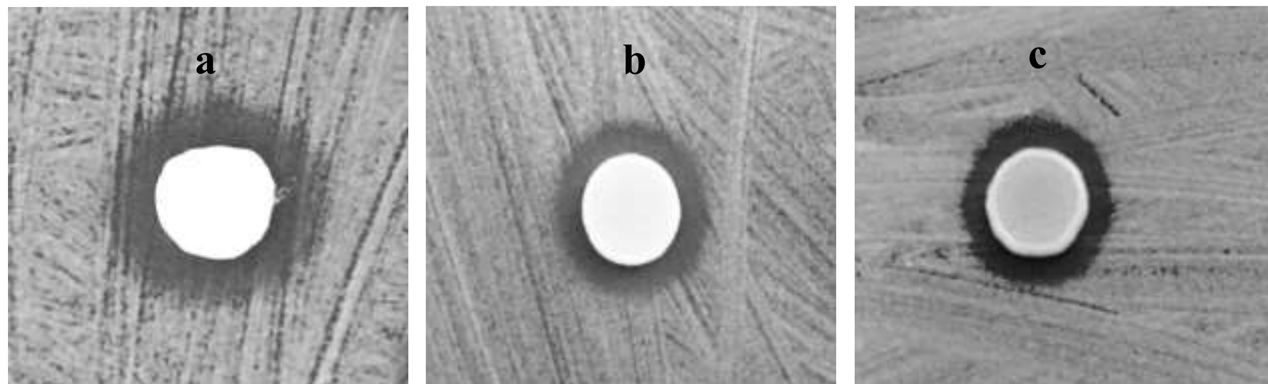

Figure 1. Clear zones formed on the assay agar seeded with pathogenic yeast. Sensitive strains: (A) *Metchnikowia bicuspidata* WCY, (B) *Candida tropicalis* Ct, and (C) *Candida albicans* YTS-03.

concentration, pH of the fermentation broth, fermentation time, and temperature as four independent variables for single-factor screening experiments were done. *M. saccharicola* was seeded at different inoculum concentrations (2 to 12%) in 500-mL Erlenmeyer flasks containing 100 mL of toxin production medium (pH 4.5) in a rotary bed shaker (140 rpm) at 28°C for 48 h. Based on the results, medium pH was varied from 3.5 to 6.5 using 0.05 M citric acid-hydrogen phosphate disodium buffer, and yeast was cultured at different temperatures (20 to 35°C) for 12 to 72 h.

Experimental design and statistical analysis

According to the results of single factor tests, a three-level-four-factor Box-Behnken Design (BBD) was applied to optimize fermentation conditions using the Design Expert (V 8.0.5) software. Inoculum concentration (X1), pH of the fermentation broth (X2), and fermentation time (X3) and temperature (X4) were the independent variables (Table 1), and killer toxin activity was used as an endpoint in the response surface tests (Su et al., 2013). Each variable was assigned three levels: Low (−1), medium (0), and high (+1).

The diameter of the inhibition zone (Y) was taken as a response of the design experiment. Full quadratic models (Bezerra et al., 2008) for killer toxin activity were established using the following mathematical equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j=2}^{4} \beta_{ij} X_i X_j \qquad 1$$

where Y is the response (diameter of inhibition zone, cm), β0 is the intercept, βi, βii, and βij are regression coefficients of the linear, quadratic, and interactive terms, respectively; and Xi and Xj represent coded independent variables. The fitted polynomial equation can be expressed as surface plots to visualize the relationships between responses and investigated parameters (Lwa et al., 2015). ANOVA was employed to evaluate the empirical mathematical model at 5% significance level and to test the significance of the difference between two or more sample mean differences. The statistical significance was considered by P-value，where the calculated P-value should be greater than the tabulated P-value to reject the null hypothesis and all the regression coefficients were 0 (Devore and Farnum, 2004).

RESULTS AND DISCUSSION

Screening of marine killer yeast against pathogenic yeast strains

The yeast strain DD21-2 isolated from river bed sediments could secrete killer toxins onto culture medium and kill pathogenic yeast *M. bicuspidata* WCY, *C. tropicalis* Ct, and *C. albicans* YTS-03 (Figure 1). The killer activity of strain DD21-2 against *M. bicuspidata* WCY was higher than that against Ct and YTS-03, as evidenced by the ratio of diameter of inhibition zone to diameter of the colony been ≥1.5 (Figure 1); therefore, *M. bicuspidata* WCY was used as a sensitive strain in further experiments.

Identification of yeast strain DD21-2

Based on colony and cell morphology (Figure 2), fermentation and carbon source assimilation (Table 2),

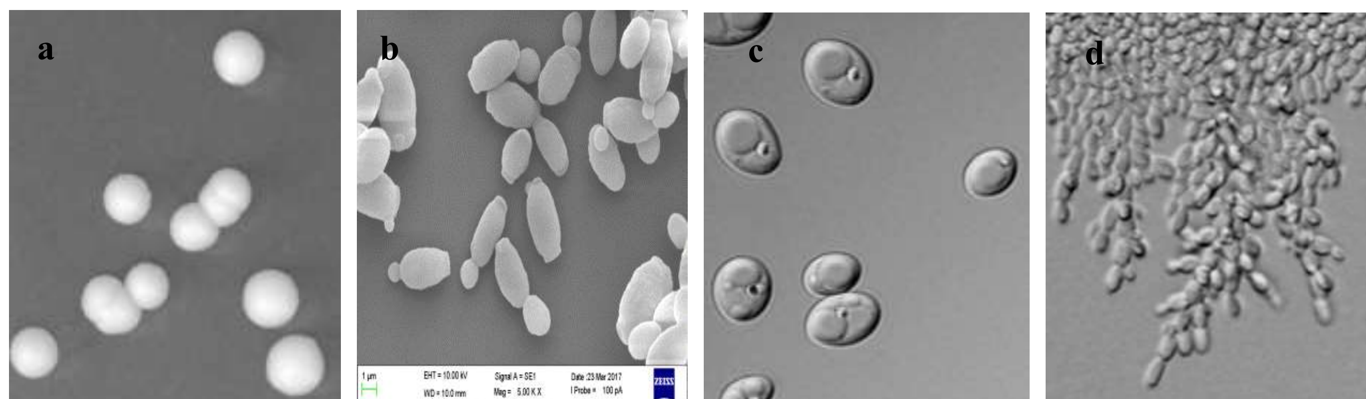

Figure 2. Images of colonies (a), scanning electron microscopy (b), budding cells (c), and pseudohyphae (d) of the *M. saccharicola* DD21-2 strain.

Table 2. Carbohydrate fermentation and assimilation by strain DD21-2.

Assimilation	Reaction	Fermentation	Reaction
Glucose	+	Glucose	+
Maltose	+	Maltose	–
Galactose	–	Galactose	l
Sucrose	+	Sucrose	–
Lactose	–	Lactose	–
Raffinose	+	Raffinose	–
Melibiose	–	Melibiose	–
Amidulin	–	Trehalose	–
Trehalose	+		
Cellobiose	+		
D-arabinose	–		
L-arabinose	–		
D-xylose	w		

+, Positive; -, Negative; l, Delayed positive; w, Weak.

and strain types of marine yeasts (Kurtzman and Fell, 2011), the yeast strain DD21-2 was related to *M. saccharicola*. Phylogenetic analysis based on ITS and the D1/D2 domain of 26S rDNA showed that many phylogenetically related yeast species were similar to the marine yeast strain and confirmed that DD21-2 was closely related to *M. saccharicola* (Figures 3 and 4). Although the phylogenetic analysis of 26S rDNA showed that Metschnikowia is similar to Candida, they differ greatly in morphology and physiology (Kurtzman and Fell, 2011; Kaewwichian et al., 2012). Moreover, the nucleotide blast on NCBI also showed that only Metschnikowia saccharicola has 100% similarity to reference sequence. Phylogenetic analysis of ITS sequencing also fully proved this point (Figure 4). Therefore, the topology of the phylogenetic in Figures 3 and 4 confirms that strain DD21-2 was *M. saccharicola* (NCBI accession number KY849584 and MF115995).

Single-factor screening experiments

Individual fermentation variables significantly influenced the activity of the yeast-derived killer toxin. Thus, killer activity was highest at an inoculum concentration of 4% (v/v; Figure 5a) and pH 5.5 (Figure 5b), but further increases inhibited toxin activity. Killer toxin activity was maximum at 54 h of fermentation (Figure 5c) and 27.5°C (Figure 5d), which is consistent with previous reports (Zhou et al., 2014; Wu et al., 2015). Therefore, the independent factors were inoculum concentration (X1, 2 to 6%, w/v), pH value (X2, 5.0 to 6.0), fermentation temperature (X3, 26 to 28°C), and fermentation time (X4, 2 to 3 days) (Table 1).

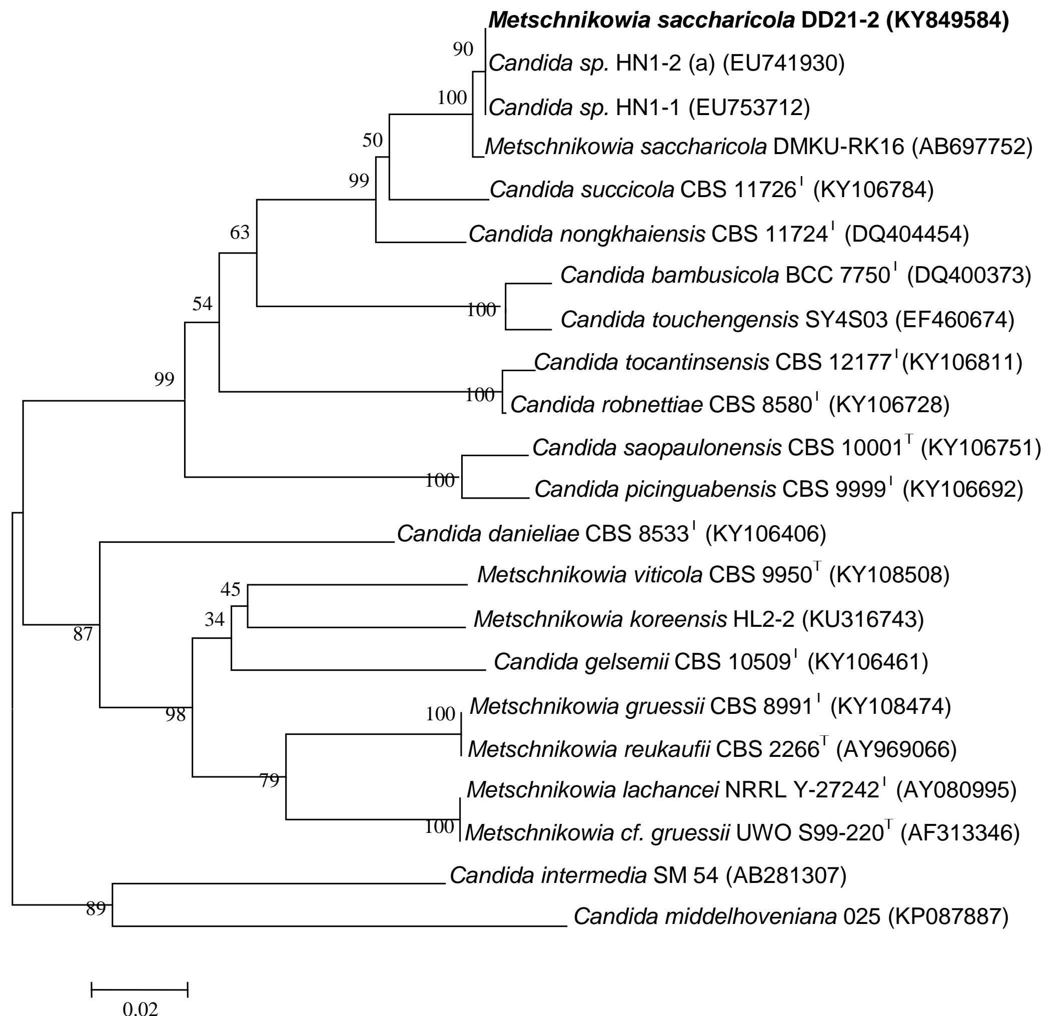

Figure 3. Phylogenetic tree of yeast strain DD21-2 and 21 of its closest relatives (type strains) constructed using neighbor parsimony analysis of the D1/D2 domain of 26S rDNA. Bootstrap support values were calculated based on 1,000 pseudoreplications; values ≥50% are shown above the branches. Strain and sequence accession numbers are shown.

RSM model analysis

Model fitting for RSM

The complete design matrix of the Box-Behnken design and the interaction effect of variables on the response are given in Table 3. A total of 29 experiments were conducted to determine the optimum fermentation conditions, and the data indicated that different levels of variables resulted in different response.

Multiple regression fitting was performed using Design Expert (V 8.0.5) (Shen et al., 2010), and full quadratic models were established using Equation 1. Through regression analysis, the relationship between inoculum concentration (X_1), pH of the fermentation broth (X_2), fermentation time (X_3), fermentation temperature (X_4), and the diameter of the inhibition zone (Y) was described by quadratic regression Equation 2:

$$Y = -45.62 + 0.62X_1 + 7.10X_2 + 1.68X_3 + 1.74X_4 + -0.08X_1X_2 + 0.11X_1X_3 - 0.01X_1X_4 + 0.10X_2X_3 - 0.02X_3X_4 - 0.03X_1^2 - 0.65X_2^2 - 0.37X_3^2 - 0.03X_4^2 \quad (2)$$

ANOVA showed that the *P* value of the model was 0.0228 (Table 4), indicating that regression was significant and that the experimental data and model were well fitted. The *P* test showed that the quadratic effect or interaction of independent variables significantly influenced the diameter of the inhibition zone. The coefficient of determination (R^2) for the quadratic model was 0.903, indicating that it was a well-fitting model that could adequately describe the relationships between the response (inhibition-zone diameter) and variables within the studied ranges. These results suggested that the empirical models for killer toxin activity provided good predictions for optimization.

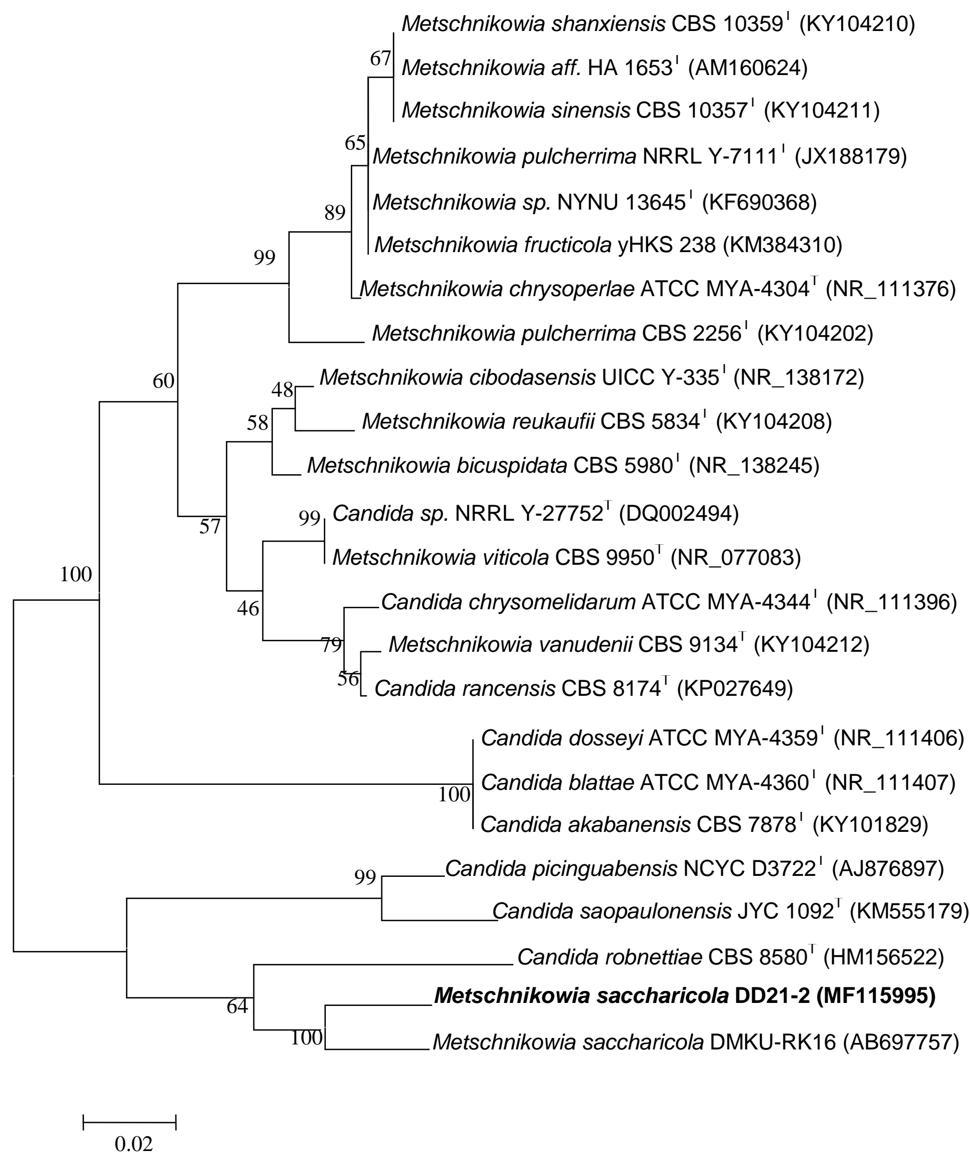

Figure 4. Phylogenetic tree of yeast strain DD21-2 and 23 of its closest relatives (type strains) based on the neighbor parsimony analysis of ITS sequences. Bootstrap support values were calculated based on 1,000 pseudoreplications; values ≥50% are shown above the branches. Strain and sequence accession numbers are shown.

Interaction effect of process variables on the diameter of the inhibition zone

The three-dimensional (3D) surfaces and the corresponding contour plots generated by the Design Expert software were used to study the effects of the parameters and their interactive effects on the killer toxin activity (Figure 6). According to the equation, the coefficients of the quadratic terms were negative, and the parabolic opening in 3D surfaces was downward, which indicated a maximum value point. Contour plots revealed significant interactive effects of the four factors on the diameter of the inhibition zone. The increment of the inhibition zone diameter with the increase of inoculum concentration and pH up to a critical point (Figure 6a) is in agreement with the fact-finding results of killer toxin' s optimal condition of Liu et al. (2012), and the ellipsoid contour plot of the inhibition-zone diameter indicated that the interaction between inoculum concentration and pH was considerable (Figure 6b).

The interaction effects of inoculum concentration and fermentation time on the inhibition zone diameter revealed that longer fermentation time produced a larger inhibition zone (Figure 6c). However, as the inoculum concentration and fermentation time increased, killer toxin activity decreased (Figure 6c), which could be

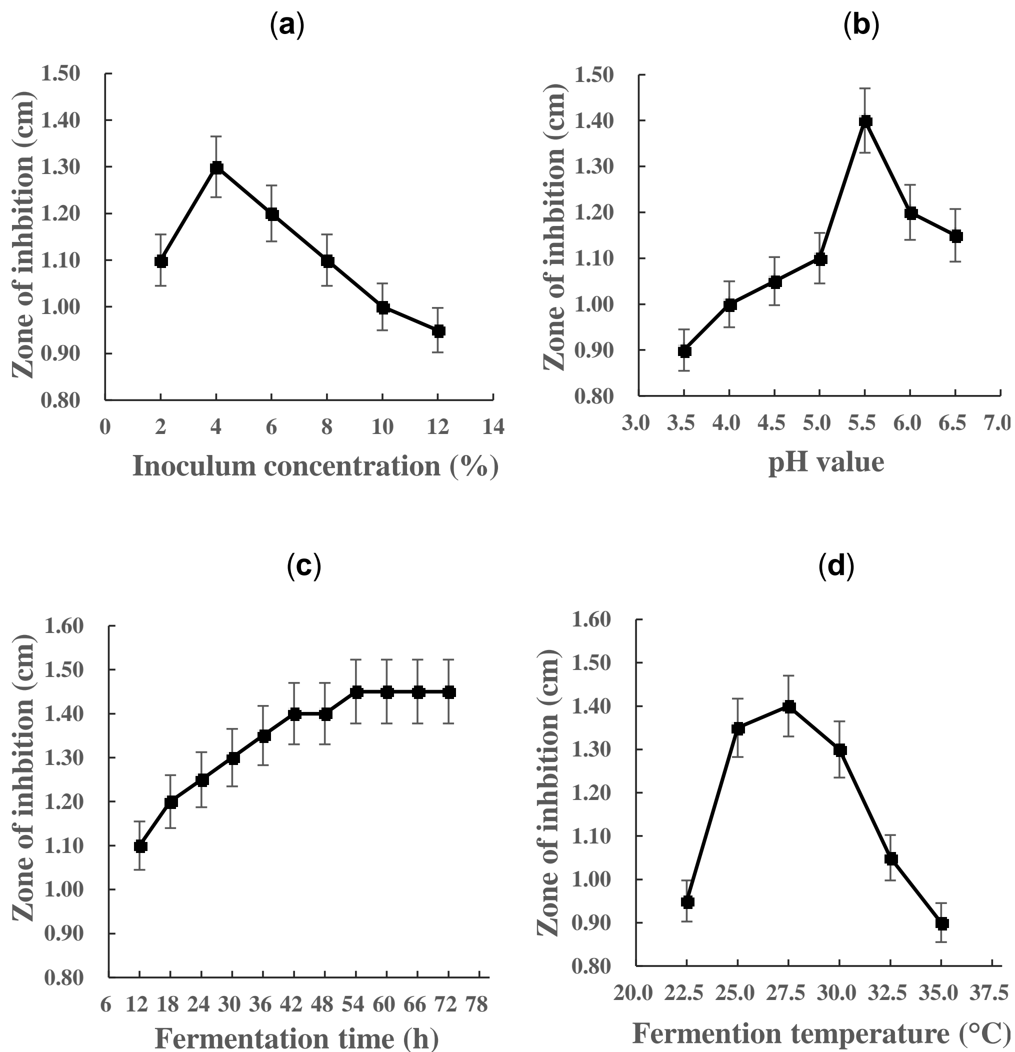

Figure 5. Effects of inoculum concentration (a), pH (b), and fermentation time (c) and temperature (d) on the activity of the killer toxin.

Table 3. Design and results of central composite tests.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response[1)]
	X_1	X_2	X_3	X_4	Y
	A: Inoculum concentration (%, v/v)	B: pH of fermentation broth	C: Fermentation time (d)	D: Fermentation temperature (°C)	Diameter of inhibition zone (cm)
1	0	0	0	0	1.45
2	1	0	1	0	1.20
3	0	1	0	1	0.95
4	0	0	-1	1	1.10
5	0	1	0	-1	1.05
6	0	0	0	0	1.40
7	0	0	1	-1	1.30
8	1	0	0	1	1.00
9	-1	0	0	1	1.30
10	0	0	0	0	1.35
11	0	0	0	0	1.35

Table 3. Contd.

12	0	0	0	0	1.40
13	0	1	1	0	1.30
14	4	-1	0	-1	1.20
15	-1	-1	0	0	1.05
16	0	1	-1	0	1.05
17	1	0	0	-1	1.15
18	0	-1	-1	0	1.10
19	-1	0	0	-1	1.30
20	-1	1	0	0	1.15
21	0	-1	0	1	1.10
22	1	-1	0	0	1.20
23	0	0	-1	-1	1.00
24	0	-1	1	0	1.25
25	-1	0	1	0	1.00
26	0	0	1	1	1.30
27	1	1	0	0	1.00
28	1	0	-1	0	1.05
29	-1	0	-1	0	1.30

[1)]Data are presented as means of 3 replications.

Table 4. ANOVA of Box-Behnken test results.

Source	Sum of squares (SS)	Degree of freedom (DF)	Mean squares (MS)	*F*-value	*P*-value[b]
Model[a]	0.4400	14	0.0320	3.05	0.0228*
A-Inoculum concentration	0.0210	1	0.0210	2	0.1793
B-pH value of fermentation	0.0130	1	0.0130	1.28	0.2771
C-Fermentation time	0.0470	1	0.0470	4.5	0.0523
D-Fermentation temperature	0.0052	1	0.0052	0.5	0.4913
AB	0.0230	1	0.0230	2.16	0.1639
AC	0.0510	1	0.0510	4.86	0.0448*
AD	0.0056	1	0.0056	0.54	0.4747
BC	0.0025	1	0.0025	0.24	0.6319
BD	0.0000	1	0.0000	0	1.0000
CD	0.0025	1	0.0025	0.24	0.6319
A^2	0.1000	1	0.1000	9.59	0.0079**
B^2	0.1700	1	0.1700	16.26	0.0012**
C^2	0.0560	1	0.0560	5.37	0.0361*
D^2	0.0900	1	0.0900	8.65	0.0107*
Residual	0.1500	14	0.0100		
Lack of fit	0.1400	10	0.0140	7.94	0.0303
Pure error	0.0070	4	0.0018		
Cor total	0.5900[c]	28[d]			

*P < 0.05, significant difference; **P < 0.01, very significant difference; [a] R^2 = 0.903; [b] Confidence level of 95%, α = 0.05; [c] Cor total sum of squares: Sum of squares total corrected for the mean; [d]Cor total degree of freedom: degree of freedom total corrected for the mean number of runs minus one.

attributed to inactivation of the killer toxin during longer fermentation (Jia, 2012). In the interaction of the inoculum concentration and fermentation temperature, the diameter of the inhibition zone increased with the increase of the inoculum concentration and temperature up to the optimum level (Figure 6e). Similarly, the

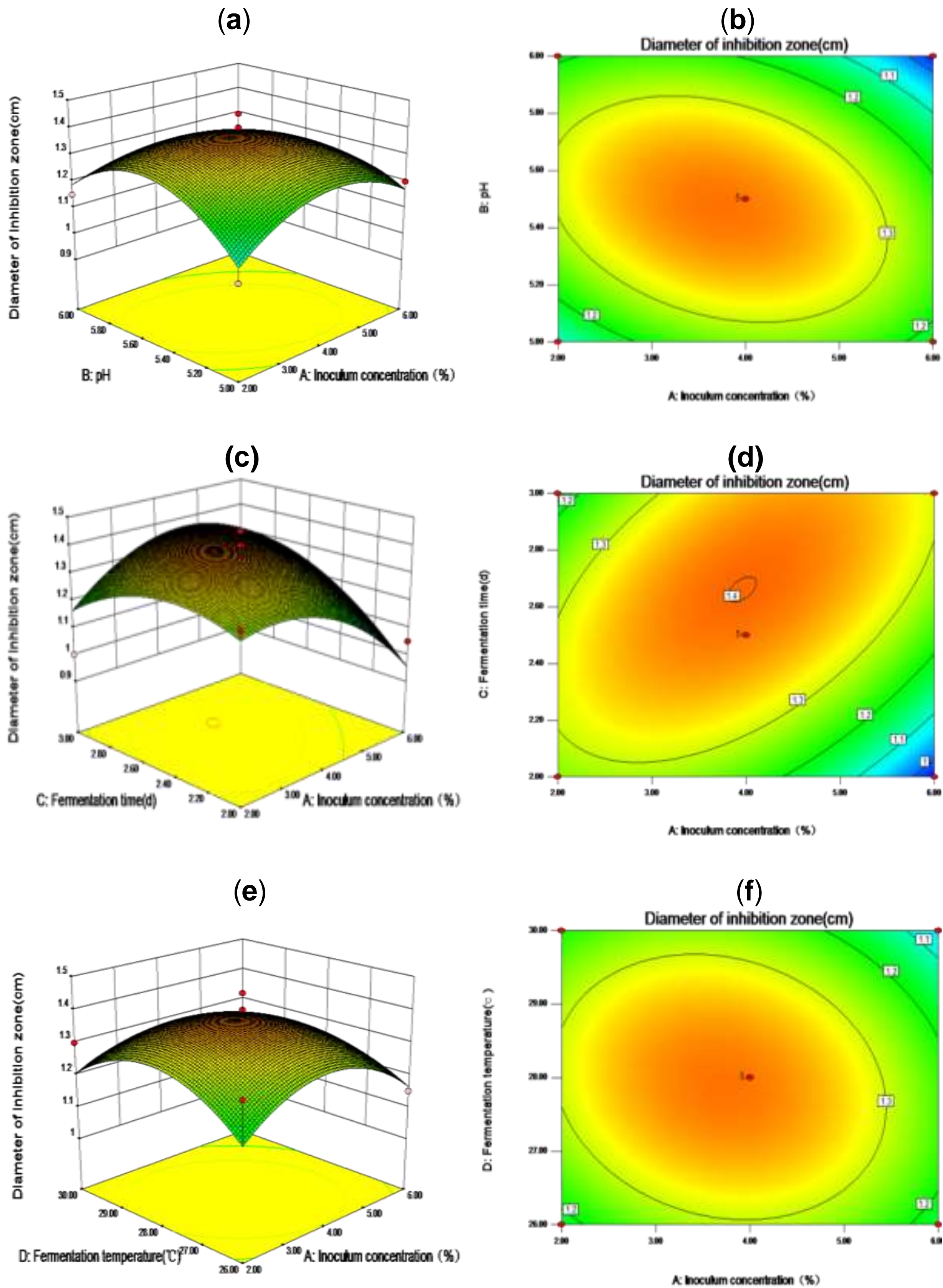

Figure 6. The three-dimensional surface map and contour map of interactions between two variables on killer toxin production by yeast strains.

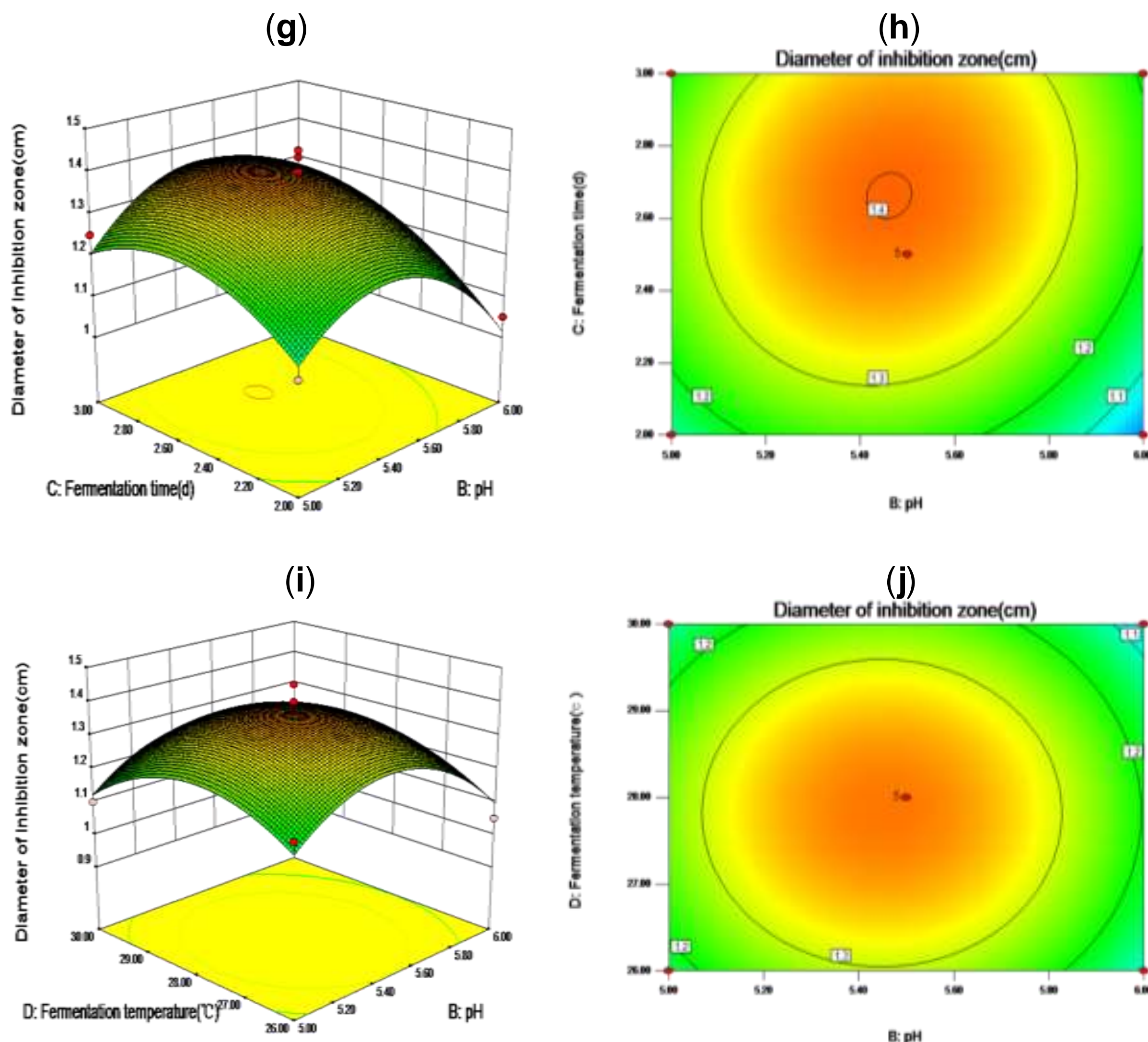

Figure 6. Contd.

interaction of pH and fermentation time significantly influenced the diameter of the inhibition zone (Figure 6g). The inhibition zone diameter was found to decrease linearly with the increase in the fermentation temperature above 27.5°C and pH above 5.5 (Figure 6i), consistent with the results obtained for *Kloeckera apiculata* KY-13c with optimal growth condition at pH 5.5 (Wu et al., 2015). Thus, high pH and fermentation temperature inhibited killer toxin activity. However, the DD21-2 strain could exert killer effects in a wider range of fermentation conditions compared with some other marine yeast, such as *Williopsis saturnus* WC91-2, which showed optimum toxin production at pH 3 to 3.5 and 16°C (Wang et al., 2012). Furthermore, fermentation time and temperature also had a significant effect on DD21-2 killer activity; the activity first increased and then decreased (Figure 6k), which was similar to that observed for *Saccharomyces cerevisiae* (Zhou et al., 2014). Thus, the interaction among the analysed factors significantly affected the inhibition-zone diameter, that is, killer toxin production.

Optimization of killer activity

Killer toxin production by *M. saccharicola* DD21-2 was optimized with RSM based on the four-factor, three-level BBD method. The results indicate that the four factors were significant in affecting killer activity, and the best fermentation conditions were determined for killer toxin production: Inoculum concentration 4.1% (v/v), pH 5.5, fermentation temperature 28°C, and fermentation time 2.7 days. These conditions were verified experimentally, and the diameters of the obtained triplicate inhibition zones were 1.45, 1.42 and 1.45 cm, which were very

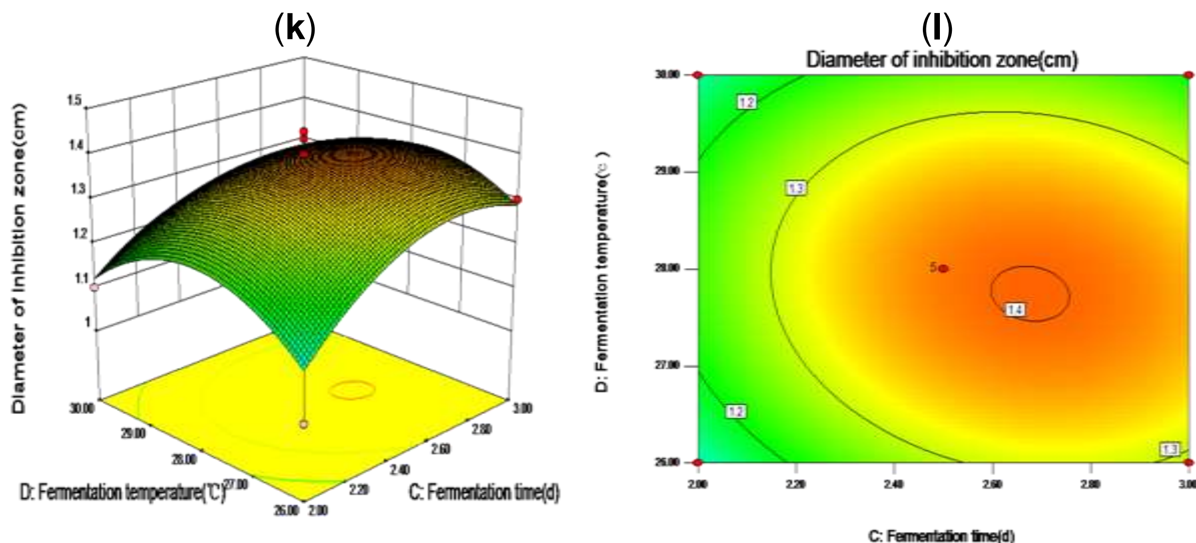

Figure 6. Contd.

close to the predicted value of 1.41 cm. The error between the predicted and experimental values (2.84%) was within the 5% level of significance, indicating that the model is acceptable and the response surface optimization of fermentation conditions is effective. Thus, higher killer toxin yields can be achieved by the aforementioned methodology and the study suggests practical directions for further research in purification and characterization, and finally provides a basis for disease prevention and control in marine aquaculture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Bandeira KF, Tininis AG, Bolzani VDS, Cavalheiro AJ (2006). Optimisation of conditions for the extraction of casearins from *Caesaria sylvestris* using response surface methodology. Phytochemical Analysis, 17:168-175.

Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76:965-977.

Chi Z, Ma C, Wang P, Li HF (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresource Technology, 98:534-538.

Chi ZM, Liu GL, Zhao SF, Li J, Peng Y (2010). Marine yeasts as biocontrol agents and producers of bio-products. Applied Microbiology and Biotechnology, 86:1227-1241.

Comitini F, Ingeniis JD, Pepe L, Mannazzu I, Ciani M (2004). *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against Dekkera/Brettanomyces spoilage yeasts. FEMS Microbiology Letters 238(1):235-240.

Çorbacı C, Uçar FB (2017). Production and Optimization of Killer Toxin in *Debaryomyces hansenii* Strains. Brazilian Archives of Biology and Technology, 60:1-11.

Devore JL, Farnum NR (2004). Applied Statistic for Engineers and Scientiest. Duxbury Press, Pasific Grove, CA, USA, pp. 394-398.

Guo FJ, Ma Y, Xu HM, Wang XH, Chi ZM (2013a). A novel killer toxin produced by the marine-derived yeast *Wickerhamomyces anomalus* YF07b. Antonie van Leeuwenhoek, 103:737-746.

Guo PH, Liu XL, Cui YP, Liao K (2013b). The value of universal fungal primers ITS1 and ITS4 in the clinical identification of filamentous fungi. Chinese Journal of Microecology, 25:922-924.

Jia RJ (2012). The preliminary study on killer toxin of yeast strain *Pichia guilliermondii* GZ1. Dissertation, Ocean University of China, Qingdao.

Kaewwichian R, Yongmanitchai W, Kawasaki H, Limtong S (2012). *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. Antonie van Leeuwenhoek, 102:743-751.

Kurtzman CP, Fell JW (2011). The yeast: a taxonomic study, 5th edn. Elsevier, Amsterdam, pp. 87-107.

Liu GL, Wang K, Hua MX, Buzdar MA, Chia ZM (2012). Purification and characterization of the cold-active killer toxin from the psychrotolerant yeast *Mrakia frigida* isolated from sea sediments in Antarctica. Process Biochemistry, 47:822-827.

Lwa HQ, Sun JC, Liu SQ (2015). Optimization of methionol bioproduction by *Saccharomyces cerevisiae* using response surface methodology. Annals of Microbiology. 65:197-205.

Magliani W, Conti S, Travassos LR, Polonelli L (2008). From yeast killer toxins to antibiobodies and beyond. FEMS Microbiology Letters 288:1-8.

Peng Y (2010). The studies on killer toxin and β-1, 3-glucanase produced by the marine yeast *Williopsis saturnus* WC91-2.

Dissertation, Ocean University of China, Qingdao.
Philliskink G, Young TW (1975). The occurrence of killer character in yeasts of variousgenera. Antonie van Leeuwenhoek, 41:147-151.
Santos A, Marquina D, Leal JA, Peinado JM (2000). (1,6)-β-D-glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. Applied and Environmental Microbiology, 66:1809-1813.
Schmitt MJ, Breinig F (2002). The viral killer system in yeast: from molecular biology to application. FEMS Microbiology Reviews 26(3):257-276.
Shen NK, Wang QY, Lu Y, Qin Y, Huang RB (2010). Enhancing ethanol production using thermophilic yeast by response surface methodology. Chinese Journal of Biotechnology, 26:42-47.
Su T, Bao B, Yan T, Zhang C, Bu Y, Wu W (2013). Response surface methodology to optimize marine microbe culture for producing fungi fibrinolytic compound. Chinese Journal of Biotechnology, 29:857-861.
Sugita T, Takashima M, Kodama M, Tsuboi R, Nishikawa A (2003). Description of a new yeast species, Malassezia japonica, and its detection in patients with atopic dermatitis and healthy subjects. Journal of Clinical Microbiology, 41:4695-4699.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGAS: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28:2731-2739.
Wang L, Yue L, Chi ZM, Wang X (2008). Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*. Diseases of Aquatic Organisms, 80:211-218.
Wang XH, Chi ZM, Yue LX, Li J, Li MJ, Wu LF (2007). A marine killer yeast against the pathogenic yeast strain in crab (*Portunus trituberculutus*) and an optimization of the toxin production. Microbiological Research, 162:77-85.
Wang XH, Jia RJ, Li J, Chi ZM (2013). Character and application of killer yeast. Biotechnology Bulletin, 4:33-38.
Wang XX, Chi Z, Peng Y, Wang XH, Ru SG, Chi ZM (2012). Purification, characterization and gene cloning of the killer toxin produced by the marine-derived yeast *Williopsis saturnus* WC91-2. Microbiological Research, 167:558-563.
Wu JH, Zhang LZ, Yang WJ, Dong F, Feng XQ (2015). Study on application of a strain of *Kloeckera apiculata* KY-13c with killer performance. Science and Technology of Food Industry 36:151-158.
Xu WJ (2005). Studies on milky disease in crab. MSc thesis, Ocean University of China, Qingdao.
Xu WJ, Xu HX, Jin HW (2003). Studies on milky disease of *Portunus trituberculatus*. Journal of Oceanology Zhejiang Province 3:209-213.
Yaakob H, Malek RA, Misson M, Jalil MFA, Sarmidi MR, Aziz R (2011). Optimization of Isoflavone Production from Fermented Soybean Using Response Surface Methodology. Food Science and Biotechnology, 20:1525-1531.
Zhou XL, Zhou YH, Gao SF, Liu HZ, Hu XX, Shu Y, Wang SP, Zeng Y (2014). Optimization of fermentation conditions and components of culture medium for *Saccharomyces cerevisiae*. Hunan Agricultural University, 8:69-72.

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Molecular evidence of *Helicobacter suis* infection in pigs in Nigeria

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***Helicobacter suis* mainly colonizes the stomach of pigs and occasionally infects humans. It is a prevalent cause of gastritis and gastric ulcers in both species. This study was designed to determine the colonization rate of *H. suis* in the stomach of pigs in Nigeria. Pig slaughter house surveys were conducted and stomach mucosa samples were collected from the fundus of the stomach of a total of 160 pigs in four locations in Nigeria (Lagos, Delta, Enugu and Plateau States). In each location, 50% of the samples were collected from stomachs with ulceration in the fundus, while the rest were from those with no gross lesions. DNA was extracted and PCR assay was conducted using standard primers. Data was analyzed by descriptive statistics and Chi-square test (p< 0.05). *H. suis* was detected in 8.75% of the samples across the four locations at a frequency of 15, 7.5, 10 and 2.5% in Lagos, Delta, Enugu and Plateau states, respectively. *H suis* was detected in 12.5 and 5% of the stomachs with ulcers and without ulcers, respectively. *H suis* colonizes pigs in Nigeria at a relatively low rate with its colonization rate being higher in stomachs with ulcers. There is need for characterization of the strains of the organism in Nigeria for a better understanding of its possible role in gastric ulceration in pigs.**

Key words: Gastric ulcer, *Helicobacter suis,* pigs, stomach.

INTRODUCTION

Numerous members of the *Helicobacter* species are adapted to the harsh acidic environment of the stomach of humans and many animal species. The colonization of the human stomach by unidentified spiral organisms was reported as early as 1889 (Konturek, 2003), while Bizzozero reported the presence of similar organisms in canine gastric mucosa in 1893 (Danon and Lee, 2001). Interestingly, the role of these bacteria in causation of gastric ulcer disease in man was not established until 1984 (Marshall and Warren, 1984). The discovery stimulated the interest of researchers in this organism, thereby leading to the discovery of increasing numbers of members of this genus and the hosts they are adapted to. *Helicobacter suis* is a zoonotic organism and it has

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Table 1. Primers used for detection of *H. suis* in stomach of the pig samples.

Species	Primers	Target gene	Sequence (5′-3′)	Annealing position	Product length (bp)
H. suis	V832f	16S rRNA	TTG GGA GGC TTT GTC TTT CCA	832 to 852	433
	V1261r		GAT TAG CTC TGC CTC GCG GCT	1261 to 1281	

been reported to colonize up to 60% of the stomach of slaughtered pigs (Grasso et al., 1996; Park et al., 2004; Kopta et al., 2010). It is associated with gastric pathologies in these animal species (Haesebrouck et al., 2009; Choi et al., 2001; Roosendaal et al., 2000). It is also the most frequently detected non-*Helicobacter pylori* Helicobacter (NHPH) species in humans with gastric disease (De Groote et al., 2005; Van den Bulck et al., 2005) where it may cause gastritis, peptic ulcer disease and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Flahou et al., 2012; Morgner et al, 2000). The risk of developing MALT lymphoma in fact seems to be higher after infection with NHPH as compared to *H. pylori* infection in humans (Stolte et al., 2002). Predisposing factors to infection may include close contacts and consumption of uncooked or undercooked pork (De Cooman et al., 2014; Pasmans and Haesebrouck, 2014). There is dearth of information on *H. suis* infection in pigs in Nigeria.

This study was therefore designed to provide information on the molecular evidence of infection and the frequency of detection of *H. suis* in pigs in Nigeria and to evaluate a possible association between its colonization and occurrence of gastric ulcers in pigs in Nigeria.

MATERIALS AND METHODS

Sampling

Stomach mucosal samples (approximately 2 cm^2) were obtained from healthy pigs presented for slaughter at four abattoirs located in Lagos, Delta, Enugu and Plateau states of Nigeria between the months of November 2016 and March 2017. The pigs were of both sexes, between the ages of 12 and 18 months from intensively managed flocks and within the weight range of 50 to 90 kg. The samples were from the fundus of the stomachs of 160 pigs (40 samples from each abattoir). 50% of the stomachs purposively sampled were with ulcers in the fundus while the rest were from stomachs without gross lesions.

Statistical analysis

Data was explored using descriptive statistics and the association between gastric ulcers and *H. suis* infection was subjected to Chi-square test ($p < 0.05$).

DNA extraction and polymerase chain reaction (PCR)

DNA from 50 mg of tissue was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ isolation kit (Zymo research corporation, USA) following the manufacturers instruction. For PCR assay, previously published primers targeting the 16S rRNA-coding gene of *H. suis* (De Groote et al., 2000; Proietti et al., 2010) were used (Table 1). The primers were manufactured by Integrated DNA Technologies, Belgium. Genomic DNA as positive controls was obtained from the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, University of Ghent, Belgium. Reactions were performed in a 25 µl consisting of 1 µl template DNA, 12.5 µl One Taq Quick-Load 2X Master Mix (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCl, 0.2 mM dNTPs, 5% glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, Xylene Cyanol FF, Tartrazine, 25 units/ml One *Taq* DNA Polymerase), 0.2 µM of each primer and 10.5 µl of nuclease free water in a G-Storm GS1 Thermal Cycler. Amplification of the 16S rRNA gene of *H. suis* was done after an initial 2 min denaturation at 95°C in 35 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 1 min with a final extension step at 68°C for 10 min and held at 4°C. The PCR products were analyzed by electrophoresis in 1.5% agarose gel containing 5 µl of GRGreen (Nucleic Acid Gel Stain, 10,000X in water) and examined by transillumination.

RESULTS AND DISCUSSION

A 433 bp fragment of the 16S rRNA-coding gene of *H. suis* was amplified in 8.75% (14/160) of the samples from four states in Nigeria (Figure 1). This included 15% of the samples from Lagos State (6/40), 7.5% from Delta State (3/40), 10% from Enugu State (4/40) and 2.5% from Plateau State (1/40). *H. suis* was detected with overall occurrence of 12.5 and 5% in the stomachs with ulceration and without gross ulceration, respectively (Figures 2 and 3). There was no significant association between the occurrence of gastric ulcers and *H. suis* infection in the stomachs (Table 2).

This study provides molecular evidence of infection and the frequency of detection of *H. suis* in pigs in Nigeria. The widespread detection of this organism in pigs calls for further search retrospectively and prospectively into its role in the causation of disease in pigs and man in Nigeria as it is an established pathogenic and zoonotic agent (Haesebrouck et al., 2009; Flahou et al., 2017).

The current findings show that *H. suis* detection rates in pigs in Nigeria are relatively low (8.75%) as compared to other reports from Europe and Asia, where it often exceeds 60% in slaughter-age pigs (Park et al., 2004; Hellemans et al., 2007). This variation in colonization may be due to the influence of climatic conditions and management factors since the influence of environmental factors on H. pylori colonization in humans has been previously documented (Kusters et al., 2006). The ease

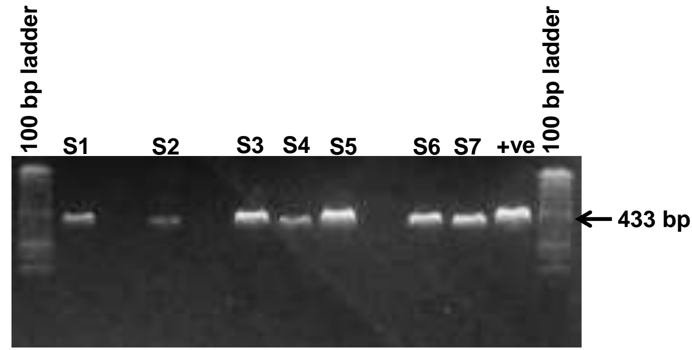

Figure 1. Gel electrophoresis image of the amplified 16sRNA gene of *H. suis* as 433 bp bands from pig stomach samples from Nigeria. S1-S6, Positive samples; +ve, positive control.

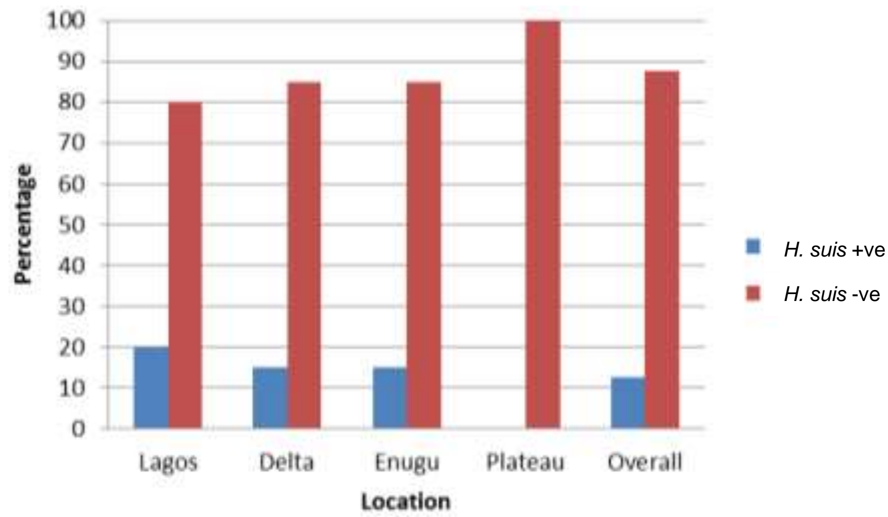

Figure 2. Frequency of detection of *H. suis* infection in stomachs of pigs with ulceration in Nigeria.

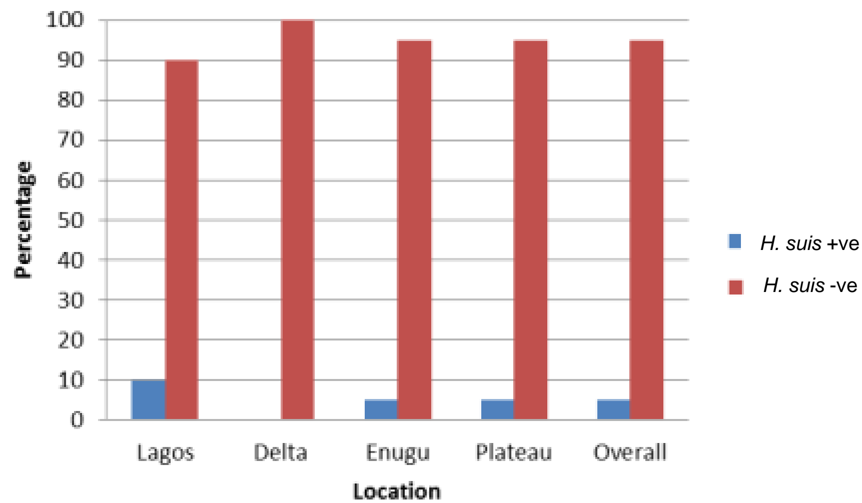

Figure 3. Frequency of detection of *H. suis* infection in stomachs of pigs without ulceration in Nigeria.

Table 2. Colonization of the stomach mucosa of pigs with *H. suis* in Nigeria.

Location		*H. suis* (+)	*H. suis* (-)	Total	Pearson Chi square value	*P*-value
Lagos	Ulcer	4	16		2.818	0.093
	No Ulcer	2	18			
		6	34	40		
Delta	Ulcer	3	17			
	No Ulcer	0	20			
		3	37	40		
Enugu	Ulcer	3	17			
	No Ulcer	1	19			
		4	36	40		
Plateau	Ulcer	0	20			
	No Ulcer	1	19			
		1	39	40		

of transfer to and adaptation of this organism in man makes it a threat to humans in close contacts with pigs as H. suis is the most prevalent NHPH affecting humans (Haesebrouck et al., 2009). This ease of transfer has been recently attributed to its primate origin (Flahou et al., 2017). H. suis is also known to cause indirect production losses in the swine industry (De Bruyne et al., 2016). In the assessment of the spread of the organism, *H. suis* was detected more frequently in stomachs with gastric ulcers in the fundus (12.5%) but there was no significant association between the occurrence of gastric ulcers and *H. suis* infection. This may infer that although the organism is associated with formation of gastric lesions in pigs, it may not be the sole factor responsible for the ulcerations observed in the fundus of the stomach of pigs in Nigeria. Other studies have conflicting reports on role of the organism in stomach pathologies in pigs (Monteiro, 2011; Queiroz et al., 1996; Roosendaal et al., 2000).

Further studies on this organism in Nigeria should include the characterization of the circulating strains and their associated lesions in pigs. In addition, the frequency of occurrence of *H. suis* in gastric tissues of humans suffering from gastric disease should also be studied as there is previous documented evidence on direct human infection with field strains of *H. suis* from pigs (Joosten et al., 2013).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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REFERENCES

Choi YK, Han JH, Joo HS (2001). Identification of novel *Helicobacter* species in pig stomachs by PCR and partial sequencing. J. Clin. Microbiol. 39:3311-3315.

Danon SJ, Lee A (2001). Other Gastric *Helicobacters* and Spiral organisms. In. Helicobacter pylori: Physiology and Genetics. Mobley HLT, Mendz GL, Hazel SL(Eds.). ASM Press, Washington D.C.. ISBN-10: 1-55581-213-9. Available at: http://www.ncbi.nlm.nih.gov/books/NBK2425/ -accessed 03/10/2016

De Bruyne E, Ducatelle R, Foss D, Sanchez M, Joosten M, Zhang G, Smet A, Pasmans F, Haesebrouck F, Flahou B (2016). Oral glutathione supplementation drastically reduces *Helicobacter* induced gastric pathologies. Sci. Rep. 6:20169.

De Cooman L, Houf K, Smet A, Flahou B, Ducatelle R, De Bruyne E, Pasmans F, Haesebrouck F (2014). Presence of Helicobacter suis on pork carcasses. Int. J. Food Microbiol. 187:73-76.

De Groote D, Ducatelle R, van Doorn LJ, Tilmant K, Verschuuren A, Haesebrouck F (2000). Detection of "Candidatus Helicobacter suis" in gastric samples of pigs by PCR: comparison with other invasive diagnostic techniques. J. Clin. Microbiol. 38:1131-1135.

De Groote D, Van Doorn LJ, Van den Bulck K, Vandamme P, Vieth M, Stolte M (2005). Detection of non-pylori *Helicobacter* species in "*Helicobacter heilmannii*"- infected humans. Helicobacter, 10(5):398-406.

Flahou B, Rossi M, Bakker J, Langermans J, Heuvelman E, Solnick J, Martin M, O'Rourke J, Ngoan L, Hoa NX, Nakamura M, Øverby A, Matsui H, Ota H, Matsumoto T, Foss DL, Kopta LA, Omotosho O, Franciosini M, Proietti PC, Guo A, Liu H, Borilova G, Bracarense AP, Lindén SK, De Bruyckere S, Zhang G, De Witte C, Smet A, Pasmans F, Ducatelle R, Corander J, Haesebrouck F (2017). Evidence for a

primate origin of zoonotic Helicobacter suis colonizing domesticated pigs. ISME J. 145:1-10.

Flahou B, Van Deun K, Pasmans F, Smet A, Volf J, Rychlik I, Ducatelle R, Haesebrouck F (2012). The local immune response of mice after Helicobacter suis infection: strain differences and distinction with Helicobacter pylori. Vet. Res. 43:75.

Grasso GM, Ripabelli G, Sammarco ML, Ruberto A, Iannitto G (1996). Prevalence of *Helicobacter*-like organisms in porcine gastric mucosa: a study of swine slaughtered in Italy. Comp. Immunol. Microbiol. Infect. Dis. 19(3):213-217.

Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, Decostere A, Ducatelle R (2009). Gastric helicobacters in domestic animalsand nonhuman primates and their significance for human health. Clin. Microbiol. Rev. 22:202-223.

Hellemans A, Chiers K, Maes D, De Bock M, Decostere A, Haesebrouck F, Ducatelle R (2007). Prevalence of "Candidatus Helicobacter suis" in pigs of different ages. Vet. Rec. 161:189-92.

Joosten M, Flahou B, Meyns T, Smet A, Arts J, De Cooman L, Pasmans F, Ducatelle R, Haesebrouck F (2013). Case Report: *Helicobacter suis* Infection in a Pig Veterinarian. John Wiley & Sons Ltd. Helicobacter, 18:392-396.

Konturek JW (2003). Discovery by Jaworski of *Helicobacter pylori* and its pathogenetic role in peptic ulcer, gastritis and gastric cancer. J. Physiol. Pharmacol. 54(Suppl. 3):23-41.

Kopta LA, Paquette, JA, Bowersock, TL, Choromanski LJ, Godbee TK, Galvin, JE, Foss DL (2010). Information of *Helicobacter suis* in pig-producing regions of North America. Conference of Research Workers in Animal Diseases, Chicago, Illinois, Dec 5-7.

Kusters JG, van Vliet AHM, Kuipers EJ (2006). Pathogenesis of *Helicobacter pylori* infection. Clin. Microbiol. Rev. 19:449-490.

Marshall BJ, Warren JR (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1311-1315.

Monteiro SAPS (2011). Prevalence and risk factors for gastric ulcers in swine. Porto: Universidade do Porto. [Online] https://repositorio-aberto.up.pt/bitstream/10216/22219/2/AbstConcl.pdf. Accessed on 10 August, 2015.

Morgner A, Lehn N, Andersen LP, Thiede C, Bennedsen M, Trebesius K, Neubauer B, Stolte M, Bayerdörffer E (2000). Helicobacter heilmannii-associated primary gastric low-grade MALT lymphoma: complete remission after curing the infection. Gastroenterology, 118:821-828.

Park JH, Seok SH, Cho SA, Baek MW, Lee HY, Kim DJ, Park JH (2004). The high prevalence of *Helicobacter* sp. in porcine pyloric mucosa and its histopathological and molecular characteristics. Vet. Microbiol. 104(3-4):219-25

Pasmans F, Haesebrouck F (2014). Presence of *Helicobacter suis* on pork carcasses. Int. J. Food Microbiol. 187:73-76

Proietti PC, Bietta A, Brachelente C, Lepri E, Davidson I and Franciosini MP (2010). Detection of *Helicobacter spp*. in gastric, fecal and saliva samples from swine affected by gastric ulceration. J. Vet. Sci. 11(Suppl. 3):221-225.

Queiroz DMM, Rocha GA, Mendes E, Moura SB, Rocha De Oliveira AM, Miranda D (1996). Association between Helicobacter and gastric ulcer disease of the pars oesophagea in swine. Gastroenterology, 111:19-27.

Roosendaal R, Vos JH, Roumen T, van Vugt R, Cattoli G, Bart A, Klaasen HL, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG (2000). Slaughter pigs are commonly infected byclosely related but distinct gastric ulcerative lesion-inducing gastrospirilla. J. Clin. Microbiol. 38:2661-2664.

Stolte M, Bayerdörffer E, Morgner A, Alpen B, Wündisch T, Thiede C, Neubauer A (2002). Helicobacter and gastric MALT lymphoma. Gut. 50(Suppl 3):19-24.

Van den Bulck K, Decostere A, Baele M, Driessen A, Debongnie JC, Burette A, Stolte M, Ducatelle R, Haesebrouck F (2005). Identification of non-*Helicobacter pylori* spiral organisms in gastric samples from humans, dogs and cats. J. Clin. Microbiol. 43(5):2256-2260.

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Interrelationships of quantitative traits and genetic variability of *Desi* type chickpea genotypes as revealed by agro-morphology and inter simple sequence repeat markers

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Plant breeders need to stay alert in developing new cultivars on a steady basis to respond to the need of ever increasing human population by exploring either natural variability present in germplasms or generating new variability. Field experiment was conducted at five locations of Western Ethiopia during the 2016/2017 main cropping season to study interrelationships and genetic variability present in Desi type chickpea genotypes using agro-morphology. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method with some minor modifications to further examine the extent of genetic variability at DNA level using inter simple sequence repeat (ISSR) markers. The analysis of variance (ANOVA) based on quantitative traits revealed highly significant genetic differences (P<0.01) among the tested genotypes for all the traits considered except number of branches per plant and number of seeds per pod. Wider genetic variations were noticed among inter cluster Euclidian distance measured based on the standardized means for the 11 quantitative traits. Grain yield, one hundred seed weight, number of pods per plant, plant height, and grain filling period had higher contributions in clustering of the genotypes. Grain yield showed significant (P≤0.01) positive association with plant height, branches number and one hundred seed weight, whereas significant negative correlation was evident with days to flowering and maturity. Higher genetic similarity (98%) and lowest similarity (0.21) were detected among tested genotypes using ISSR markers. Using morphological and molecular evaluation, the existence of genetic variability was evident, indicating the likelihood of hybridization in genetically dissimilar varieties.

Key words: Chickpea (*Cicer arietinum* L.), principal component analysis, cluster, correlation, genetic similarity.

INTRODUCTION

Pulses are important local food crops in Ethiopia that cannot be overemphasized because of their significant role in sustaining food security, balancing ecosystem, and generating revenue in Ethiopia (Keneni et al., 2012). Among pulses, chickpea (*Cicer arietinum* L.; 2n = 2x = 16), one of the ancient crops in Ethiopia, is self-

pollinating grain legume of Near -East origin and currently grown in over 50 countries of the world. Archaeological evidence from Lalibela caves discovered seed samples dated to Iron Age (Dombrowski, 1970).

Currently, with ever increasing population growth, the need for food in terms of quantity, quality and other products of plants are constantly increasing, whereas diminishing of agricultural lands, emergence of new pathogens, climate change, also consumer needs and preferences present increasing risks to crop production. As this increasing population requires food to sustain, the only way for plant breeders to stay alert in developing new cultivars on a steady basis to respond to these changes resides in exploring either natural variability present in germplasm or generating new variability by using different breeding methods such as crossing, inducing mutations and gene transfer (Kharkwal and Shu, 2009).

Along the line, geneticists devoted their time in developing different mechanisms for detection of genetic variations. Morphological and agronomical characterization are the earliest methods employed and still take up high attention to study genetic variability, even though it is exposed to strong environmental influence (Ismail et al., 2001). On the other hand, molecular methods can be used for the determination of genetic variation, excluding all environmental influences, by detecting the variation at the DNA level (Bayraktar and Dolar, 2009). Among DNA markers, inter simple sequence repeat (ISSR) was preferred in genetic diversity, phylogeny, genomics and evolutionary studies due to their easiness to use, cost effectiveness, and their multiple amplification products (Reddy et al., 2002).

The ultimate goal of the breeders is to improve yield. However, as yield and its components are polygenic in nature they are highly subjected to interaction of many other traits which influence yield directly or indirectly. Therefore, improvement aimed at single traits would be deceptive, especially when trait under consideration is a polygenic trait. For this reason, it is desirable to examine the comparative contributions of various interacting components to yield. Thus, the association among yield concurrently with its component parameters provides the basis for the effective selection in breeding schemes, than selection that is merely based on yield (Kumar and Shukla, 2002). The simple correlation analysis is one of the effective statistical tools used to assess the interaction between two traits. Besides, principal component analysis (PCoA) was used to differentiate major contributing traits to the total underlying variation in the correlation coefficients by reducing the large set of variables to a single set (Lule et al., 2012).

Therefore, the present study was conducted with the objective of estimating interrelationships among quantitative traits and genetic variability present in Desi type chickpea genotypes using agro-morphology and ISSR.

MATERIALS AND METHODS

Plant materials and sites description

Field experiment was conducted at five locations *viz.,* Shambu (09° 32'N 037° 04'E), Hawa Galan (08° 38' N 034° 50'E), Mata (08° 34' N 034° 44'E), Alaku Belle (08° 37'N 034° 42'E) and Badesso (034° 42'E 034°47'E), Western Ethiopia, during the 2016/2017 main cropping season. A total of 16 Desi type chickpea varieties *viz.,* 8 cultivars released over three decades, 1 local variety and 7 advanced lines collected from Debre Zeit Agricultural Research Center (DZARC) were used (Table 1). The experiment was laid out in a randomized complete block design with three replicates and with plot size of 3 m length and 1.8 m width. All other crop management practices and recommendations were applied uniformly to all varieties as recommended for the crop.

Data collection and statistical analysis

Days to 50% flowering, days to 90% maturity, grain filling duration, number of pods per plant, number of seeds per pod, plant height, number of branches per plant, one hundred seed weight, and grain yield data were collected based on chickpea (*C. arietinum* L.) descriptor (IBPGR, ICRISAT and ICARDA, 1993) and were subjected to analysis using appropriate software. Genomic DNA was extracted using the CTAB method (Borsch et al., 2003) with some minor modifications. Clustering was performed using MINITAB14 software (Minitab Inc., 2003). Correlation coefficients between characters were estimated based on the standard procedure. Principal component analysis (PCA) was computed by using MINITAB14 software (Minitab Inc., 2003), to identify the most important contributing traits, to the total variations observed among the genotypes.

Molecular data scoring was performed as discrete statistics, using a binary matrix as "0" for the absence of a band, "1" for the presence of a band, and "?" for the ambiguous band. POPGENE version 1.32 (Yeh et al., 1999) was used to investigate and characterize genetic diversity in terms of a number of polymorphic loci, percent of polymorphism, means of Nei's genetic diversity and Shannon index (Nei, 1972). NTSYS- pc version 2.02 (Numerical Taxonomy System; Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software programs were used to compute Jaccard's similarity coefficient as:

$$s_{i_j} = \frac{a}{a + b + c}$$

Where, ‘a' is the total number of bands shared between individuals i and j, ‘b' is the total number of bands present in individual i, but not in individual j and ‘c' is the total number of bands present individual j, but not in individual i.

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Table 1. Passport description of the tested genotypes.

Genotypes Codes	Genotypes names	Status	Year of release
G1	Akaki	Released	1995
G2	Dalota	Released	2013
G3	Dimtu	Released	2012
G4	Dubie	Released	1978
G5	Local	Local variety	-
G6	Mariye	Released	1985
G7	Minjar	Released	2010
G8	Natoli	Released	2007
G9	Teketay	Released	2013
G10	DZ-2012-CK-0032	Advanced line	-
G11	DZ-2012-CK-0034	Advanced line	-
G12	DZ-2012-CK-0233	Advanced line	-
G13	DZ-2012-CK-0237	Advanced line	-
G14	DZ-2012-CK-0312	Advanced line	-
G15	DZ-2012-CK-0313	Advanced line	-
G16	DZ-2012-CK-20113-2-0042	Advanced line	-

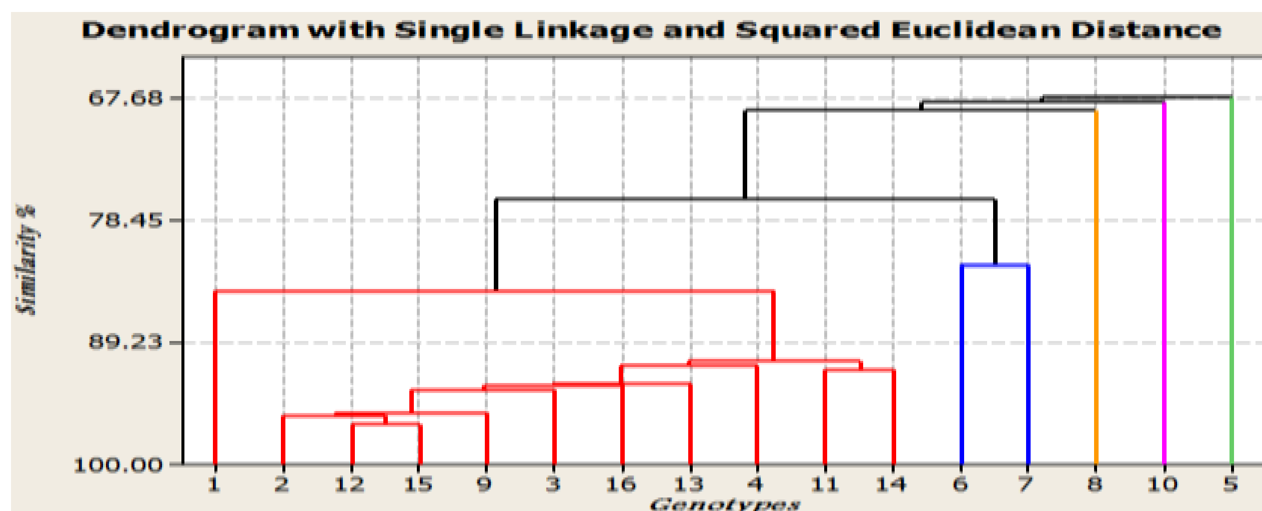

Figure 1. Dendrogram showing the similarities among 16 chickpea genotypes for 11 quantitative traits.

Neighbor Joining (NJ) and Unweighted Pair Group Method with Arithmetic Averages (UPGMA) clustering method was computed by using Free Tree 0.9.1.50 Software and NTSYS- pc software, respectively. Principal coordinate analysis (PCoA) was figured using PAST version 1.18 (Hammer et al., 2001) software based on Jaccard's coefficient similarity for further examining the patterns of variation among individual genotypes.

RESULTS AND DISCUSSION

Cluster and distance analyses

The D^2 values based on the pooled mean of genotypes resulted in classifying the 16 genotypes into five distinct clusters. Two clusters and three solitary groups were formed at 80% similarity level (Figure 1). This result agrees with the report of Malek et al. (2014) who compounded 31 mutant soybean genotypes into five clusters using nine morphological traits. It is usually expected that characters with larger absolute values, closer to unity within the first principal component influence the clustering more than those with lower absolute values, closer to zero (Chahal and Gosal, 2002). The mean value of the nine quantitative characters in each cluster is presented in Table 2.

Inter cluster Euclidean distance measured based on

Table 2. Distances between cluster centroids.

Correlation	Cluster1	Cluster2	Cluster3	Cluster4	Cluster5
Cluster1	0.000	6.379	4.110	5.539	4.480
Cluster2		0.000	5.420	7.940	5.537
Cluster3			0.000	5.390	5.340
Cluster4				0.000	7.131
Cluster5					0.000

Table 3. Differences among the five clusters of sixteen Desi chickpea genotypes for mean agronomic characters' performance.

Character	Clusters					Mean
	C1	C2	C3	C4	C5	
Days to flower	62.63	64.54	71.6	60.67	60.07	63.90
Days to mature	139.06	136.94	135.93	138.60	138.07	137.72
Grain filling period (days)	76.43	72.4	64.33	77.93	78	73.82
Nº of pods per plant	28.36	40.89	33.41	31.97	41.74	35.27
Nº of seeds per pod	1.21	1.25	1.28	1.18	1.47	1.28
Plant height (cm)	53.30	48.23	46.68	52.8	46.81	49.56
Nº of branches	4.09	4.74	3.89	4.13	4.25	4.22
Hundred seed weight (g)	31.76	24.65	32.4	29.05	14.01	26.37
Grain yield (ton ha^{-1})	1.64	1.715	2.04	1.85	1.35	1.72

the standardized means for the eleven quantitative traits revealed relatively wider genetic variation (D^2) between cluster two and cluster four, followed by cluster four and cluster five (Table 2). This indicates that the hybridization between the genotypes of these clusters would yield desirable segregates, with accumulation of favorable genes in the segregating generations. The greater distance between the two clusters shows wider genetic distance between their genotypes.

The number of member genotypes varied from cluster to cluster. The first cluster accommodated the largest number of genotypes (11 genotypes or 69%) than any other cluster. The group is distinguished by lateness in days to maturity, high plant height and hundred seed weight. Cluster (C2) with 2 members or 12% of the total population constituted two chickpea varieties (G6 and G7) that are distinguished by their high number of pods per plant and seeds per pod. Among the solitary groups, genotype G8 is characterized by its high yield, late flower, early maturity and short grain filling period; genotype G10 showed intermediate hundred seed weight and early flower, whereas genotype G5 showed early flowering, late maturity and long grain filling period tied with inferior yield (Table 3).

Principal component analysis

As suggested by Johnson and Wichern (1988), considering the first four components with an eigenvalue greater than one, it explains a substantial portion of the total variations and is constituted by values of 36, 25.8, 14.8 and 11.8%, respectively (Table 4). Grain yield, hundred seed weight, number of pods per plant, plant height, and grain filling period showed greater absolute values of eigenvectors, either in the first, second and/or third principal components. This indicated that these traits had higher contributions in clustering of these genotypes, according to their class. In other word, selection efforts based on these traits are effective and productive.

Buta and Mekbib (2011) reported contribution of number of seeds per plant and number of pods per plants in differentiating chickpea varieties. Similar report was made by Keneni et al. (2012) indicating that traits such as pod and seed setting, dry matter accumulation, nitrogen and grain yields, growth rate and grain production efficiency, along with nitrogen fixation had higher contribution in clustering of chickpea genotypes. According to Nawab et al. (2013), number of pods per plant, seeds per plant, biological yield, and grain yield had great contribution in clustering chickpea genotypes. In disparity to this, other traits such as days to flowering, days to maturity, seeds per pod and harvest index contribute more in fourth and fifth principal components. Nevertheless, the gross contribution of variation accounted on these coordinates is as low as 16.8% and therefore, had a very restricted role in clustering these genotypes.

Table 4. Eigenvalue, proportion and cumulative variances and eigenvectors on the first five principal components for 10 agronomic traits in sixteen chickpea genotypes.

Parameter	Principal component (PCs)				
	PC1	PC2	PC3	PC4	PC5
Eigenvalue	3.6030	2.5787	1.4818	1.1754	0.4981
Proportion (%)	36	25.8	14.8	11.8	5
Cumulative (%)	36	61.8	76.6	88.4	93.4
Variable	**Eigenvector**				
Days to flowering	-0.094	-0.497	0.141	0.484	-0.009
Days to maturity	-0.389	-0.065	0.302	0.453	0.310
Grain filling period (days)	-0.294	0.465	0.156	-0.058	0.323
No. of pods per plant	0.424	-0.126	0.440	-0.113	0.000
No. of seeds per pod	0.369	0.041	0.012	0.518	-0.110
Plant height (cm)	0.442	0.073	0.189	-0.217	-0.317
No. of branches	0.045	-0.058	0.783	-0.208	-0.068
Harvest index	0.005	-0.470	-0.102	-0.323	0.712
Hundred seed weight (g)	-0.468	-0.141	-0.044	0.128	-0.121
Grain yield (ton ha^{-1})	0.451	-0.514	-0.096	-0.255	-0.402

Table 5. Pearson correlation coefficient for major quantitative traits of sixteen chickpea genotypes.

Traits	DF	DM	NPPP	SPP	PH	BRN	HSW	GYLD
D2F	1	0.12	0.12	-0.07	-0.46***	-0.23***	-0.23***	-0.44***
D2M		1	0.02	-0.08	-0.15**	-0.37***	0.01	-0.48***
NPPP			1	0.04	-0.02	0.12	-0.38***	0.06
SPP				1	0.137*	0.088	-0.14*	0.07
PH					1	0.297***	0.34***	0.41***
BRN						1	0.04	0.32***
HSW							1	0.32***
GLYD								1

DF=Days to flower; DM = Days to mature; NPPP=Number of pods per plant; SPP=Seeds per pod; PH =Plant Height (cm); BRN=Branch Number; HSW=Hundred seed weight (g); GYLD=Grain yield (ton ha^{-1}).

Correlations between characters

In fact, grain yield and its components are polygenic traits that are strongly influenced by the environment and therefore, it is important to analyze the data for the relative contribution (correlation/association) of various components to yield performance. Grain yield exhibited significant ($P \leq 0.01$) negative correlation with days to flowering (r = -0.44) and maturity (r = -0.48). Similar results were reported from moisture stressed areas in Australia, where higher yields were manifested by genotypes characterized by early flowering and rapidly setting pods (Berger et al., 2003). Nevertheless, this scenario may not be the case in areas where moisture is available, thereby, supporting late flowering and maturing genotypes to exploit the available moisture and perform better than early genotypes (Wallace and Yan, 1998). Tesfamichael et al. (2015) tested twenty chickpea genotypes in areas holding long and short rain across Kenya and reported that the genotypes gave better yield under long rain areas, than the short rain areas.

Grain yield showed significant positive association with plant height (r = 0.40), branches number (r=0.33) and hundred seed weight (r=0.32). Numerous findings indicated that any positive increase of plant height, branches number and hundred seed weight improve grain yield of chickpea (Saleem et al., 2002; Raval and Dobariya, 2003; Toker, 2004; Farshadfar and Farshadfar, 2008; Ali et al., 2009; Zali et al., 2011; Malik et al., 2010; Keneni et al., 2012). Among these traits, plant height had the strongest association with the grain yield, suggesting that this trait may be important yield predictors and perhaps it is the most important for yield improvement in chickpea (Table 5). Even if it was not significant, grain yield was positively associated with the number of pods per plant and the seeds number per pod. A weak

association between grain yield and these characters may have emanated from the impact of environmental factor that was expected when genotypes are grown in a diverse environment. Generally, it is evident that traits that had positive and significant correlation with grain yield can be used by plant breeders for indirect selection of high yielding genotypes and enables them to shape breeding programs.

Genetic diversity analysis based on ISSR markers

ISSR-PCR amplification and banding pattern

A total of nine ISSR primers were initially screened for molecular analysis to depict genetic diversity, among which only three primers that produced relatively clear amplification, and explicit bands were selected. The scope of all amplified bands ranged from about 300 to 2000 base pairs. A total of 25 bands were scored from three primers *viz.* nine for UCB-834, eight for UCB-810 and UCB-880 primers. Buta and Mekbib (2011) also reported 38 bands using four primers. Amplification of one hundred and fifty-five bands from fifteen individuals of three genuine species of rhubarb using the 15 ISSR primers was reported (Wang, 2011). Twenty-one polymorphic loci were found in three assessed primers with a total of 84% polymorphisms. One hundred and seven polymorphic loci were stated in 11 assayed primers with the total of 90% polymorphisms in faba bean (Behailu, 2016). In the present study, Nei's gene diversity and Shannon's Information index with values of 0.29±0.19 and 0.44±0.26 were noticed among the chickpea genotypes tested. Parallel with this result, Nei’s gene diversity and Shannon index with values of 0.27 and 0.41 were also reported among Ethiopian faba bean varieties (Behailu, 2016). The mean number of alleles and effective number of alleles were documented to be 1.84±0.37 and 1.51±0.39, respectively.

Genetic similarity

Genetic similarities were calculated according to the method developed by Jaccard (1908). UPGMA and Neighbor Joining analysis were used to construct dendrogram for desi type chickpea genotypes by using Jaccard’s similarity coefficient. The average of Jaccard genetic similarity among chickpea cultivars and advanced lines varied from 0.21 to 0.98. Lower and upper values of genetic similarity coefficient indicate the minimum and maximum similarity, respectively. Higher genetic similarity (98%) was detected between G1 and G2, G6 and G10, and G13 and G14, whereas lowest similarity (0.21) was between G3 and G15. Above all, a genetic similarity coefficient indicates that varieties or groups that are genetically similar could not be used in the hybridization, as they are not fine in attaining the required traits. A similarity coefficient that varied from 0.41 to 0.97 was also attested among 19 released chickpea varieties (Buta and Mekbib, 2011). Jaccard’s similarity coefficients which ranged from 0.01 to 0.90 were also reported among 125 cultivars of chickpea (*C. arietinum* L.) of Indian origin, using ISSR markers (Aggarwal et al., 2015).

Cluster analysis based on ISSR DNA marker

The chickpea genotypes were grouped into five clusters in a UPGMA-based dendrogram. In the first cluster, G15 and G16 were encompassed, whereas a local landrace (G5) was clustered in the second cluster. G3 and G12 were grouped in the third cluster, whereas G13, G14, G7, G9, G8, G10, and G6 were grouped in the fourth cluster. G1, G2, G4, and G9 were placed in the fifth cluster (Figure 2). The clustering pattern observed indicated that the released varieties, advanced lines and local landraces showed clear separation, except in few cases, where released varieties and advanced lines were amalgamated. The current clustering pattern observed in this study agrees with Aggarwal et al. (2011) in their studies of genetic diversity in 115 chickpea genotypes from India using ISSR and assembled the individuals into five clusters, depending on their genetic similarity. Similarly, Gautam et al. (2016) reported three broad clusters of chickpea genotypes, obtained from dendrogram of 13 chickpea accession, including accessions from Ethiopia.

Neighbor-Joining based clustering grouped the 16 Desi type chickpea genotypes into three major clusters from the main node (Figure 3) comprising, seven, three, and six chickpea genotypes in the first, second, and third cluster, respectively. In the first cluster, one advanced pipeline, and six released varieties were included. In the second cluster, a local variety and two released varieties were grouped and in the third cluster, two released varieties and four advanced pipeline genotypes were grouped together.

Principal coordinate analysis

In the first three coordinates of the PCoA, eigenvalues of 1.95, 1.61 and 1.16 with variance of 18.24, 15.11 and 10.88%, respectively, were recorded. Except in a few cases where there were intermixed clusters, the clustering pattern observed in two-dimensional PCoA fully agrees with that of the clustering pattern observed in UPGMA and Neighbor Joining clustering (Figure 4). Behailu (2016) also reported the resemblances of clustering pattern among UPGMA, NJ and PCoA in faba bean varieties.

Conclusion

Analyzing the patterns and causes of phenotypic and

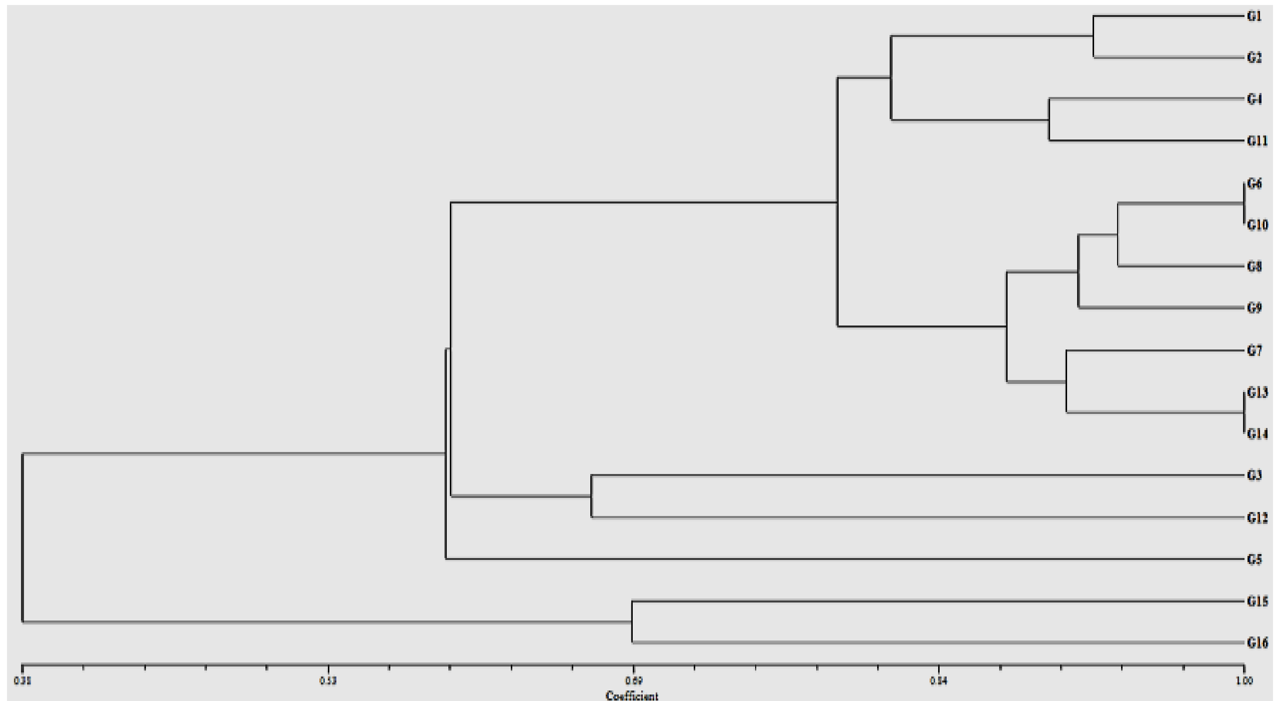

Figure 2. Dendrogram for 16 Desi type chickpea genotypes based on UPGMA analysis of amplified bands by ISSR primers.

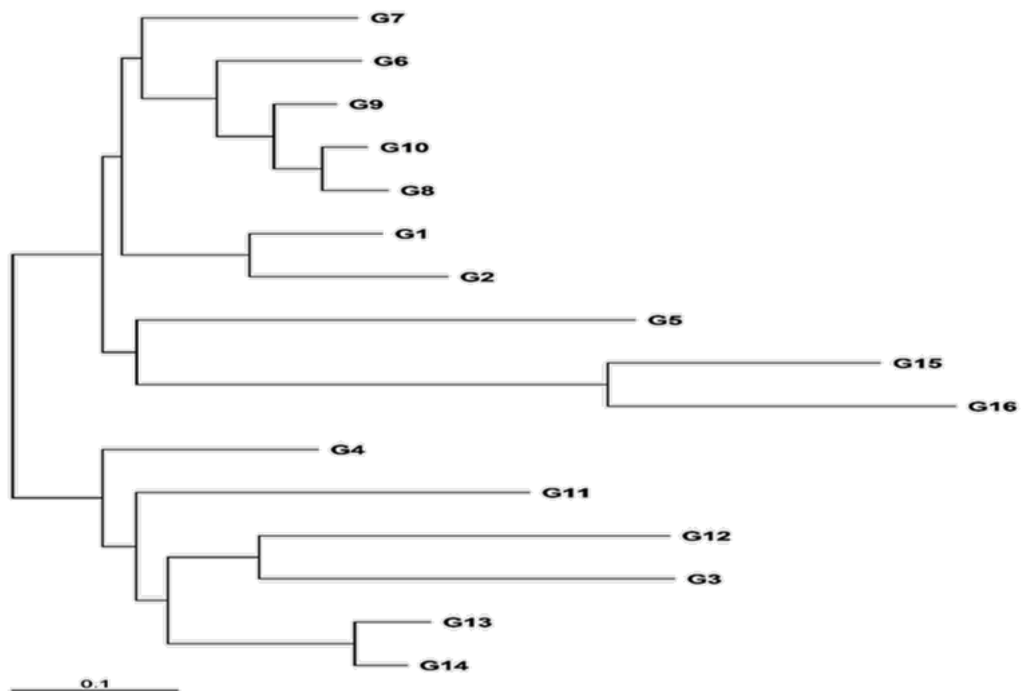

Figure 3. Neighbor-Joining clustering of 16 Desi type chickpea genotypes in Ethiopia.

genetic variation help us to understand variability in plants, to predict their responses to changing environmental conditions and thereby to plan proper breeding schemes for crop improvement. Morphological and molecular evaluation of the chickpea genotypes in this study showed the existence of genetic variability,

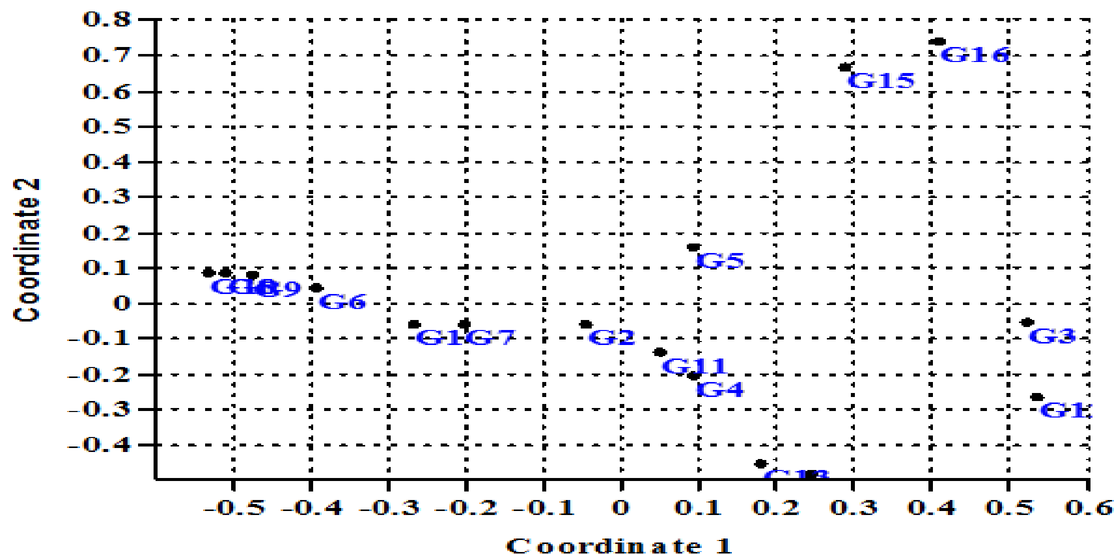

Figure 4. Two-dimensional representation of principal coordinate analysis of genetic relationships among 16 chickpea genotypes inferred from similarity matrix using the Jaccard's index.

indicating the likelihood of hybridization in genetically dissimilar varieties. Plant height, branches number and hundred seed weight had the strongest association with the grain yield, suggesting that these traits may be important yield predictors and perhaps the most important for yield improvement in chickpea.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Aggarwal H, Rao A, Kumar A, Singh J, Rana JS, Naik PK, Chhokar V (2015). Assessment of genetic diversity among 125 cultivars of chickpea (*Cicer arietinum* L.) of Indian origin using ISSR markers. Turkish Journal of Botany, 39(2):218-226.

Aggarwal H, Rao A, Rana JS, Singh J, Kumar A, Chhokar V, Beniwal V (2011). Inter simple sequence repeats reveal significant genetic diversity among chickpea (*Cicer arietinum* L.) genotypes. Journal of Plant Sciences, 6(5):202.

Ali MA, Nawab NN, Abbas A, Zulkiffal M, Sajjad M (2009). Evaluation of selection criteria in *Cicer arietinum* L. using correlation coefficients and path analysis. Australian Journal of Crop Science, 3(2):65.

Bayraktar H, Dolar FS (2009). Genetic diversity of wilt and root rot pathogens of chickpea, as assessed by RAPD and ISSR. Turkish Journal of Agriculture and Forestry, 33(1):1-10.

Behailu M (2016). Genetic diversity study of Ethiopian faba bean (*Vicia faba* L.) Varieties using phenotypic traits, ISSR and SNP markers (MSc Thesis, Addis Ababa University).

Berger J, Turner NC, French RJ (2003). The role of phenology in adaptation of chickpea to drought. In. M. Unkovich and O'Leary (eds.). Solutions for a better environment. Proceedings of the 11th Australian Agronomy Conference.

Borsch T, Hilu KW, Quandt D, Wilde V, Neinhuis C, Barthlott W (2003). Noncoding plastid *trnT-trnF* sequences reveal a well resolved phylogeny of basal angiosperms. Journal of Evolutionary Biology. 16:558-576.

Buta Z, Mekbib F (2011). Genetic Diversity of Elite Chickpea (*Cicer arietinum* L.) Varieties Using Morphological and Inter Simple Sequence Repeat Markers (Master's Thesis, Haramaya University).

Chahal GS, Gosal SS (2002). Principles and procedures of plant breeding: biotechnological and conventional approaches. Alpha Science International Limited. 600 p.

Dombrowski J (1970). Preliminary report on excavations in Lalibela and Natchabiet Caves, Begemeder. In. Annales d'Ethiopie Persée-Portail des revues scientifiques en SHS. pp. 21-29.

Farshadfar M, Farshadfar E (2008). Genetic variability and path analysis of chickpea (*Cicer arientinum* L.) landraces and lines. Journal of Applied Sciences, 8(21):3951-3956.

Gautam AK, Gupta N, Bhadkariya R, Srivastava N, Bhagyawant SS (2016). Genetic Diversity Analysis in Chickpea Employing ISSR Markers. Agrotechnology, 5(152):2.

Hammer O, Harper DA, Ryan PD (2001). PAST: Paleontological statistics software package for education and data analysis. Paleontological Electronic, 4:9.

IBPGR, ICRISAT and ICARDA (1993). Descriptors for Chickpea (*Cicer arietinum* L.). IBPGR, Rome Italy/ ICRISAT, Patancheru, India/ICARDA, Aleppo, Syria.

Ismail AA, Khalifa MA, Hamam AK (2001). Genetic studies on some yield traits of durum wheat. Asian Journal of Agricultural Science, 32:103-109.

Jaccard P (1908). Novellus recherche Sur la distribution florale. Bulletin de la Société vaudoise des Sciences Naturelles, 44:223-270.

Johnson RA, Wichern DW (1988). Multivariate Statistics: A Practical

Approach. Pearson Education, Inc. Pearson Prentice Hall, Upper Saddle River, New Jersey. pp. 164-174.

Keneni G, Bekele E, Imtiaz, Dagne K, Getu I, Assefa F (2012). Genetic Diversity and Population Structure of Ethiopian Chickpea (*Cicer arietinum* L.) Germplasm Accessions from Different Geographical Origins as Revealed by Microsatellite Markers. Plant Molecular Biology Reporter, 30(3):654-665.

Kharkwal MC, Shu QY (2009). The role of induced mutations in world food security. Q. Y. Shu, Ed. In. Induced Plant Mutations in the Genomics Era, Food and Agriculture Organization of the United Nations, Rome, Italy. pp. 3-38.

Kumar P, Shukla RS (2002). Genetic analysis for yield and its attributed traits in bread wheat under various situations. Jawaharlal Nehru Krishi Vishwa Vidyalaya Research Journal, 36:95-97.

Lule D, Tesfaye K, Fetene M, De Villiers (2012). Multivariate analysis for quantitative traits in finger millet (*Eleusine coracana* subsp. Coracana) population collected from Eastern and Southeastern Africa: detection for patterns of genetic diversity. International Journal of Agricultural Research, 7(6):303-314.

Malek MA, Rafii MY, Afroz MS, Nath UK, Mondal, MMA (2014). Morphological Characterization and Assessment of Genetic Variability, Character Association, and Divergence in Soybean Mutants. Scientific World Journal.

Malik SR, Bakhsh A, Asif MA, Iqbal U, Iqbal SM (2010). Assessment of genetic variability and interrelationship among some agronomic traits in chickpea. International Journal of Agriculture and Biology, 12(1):81-85.

MINITAB Inc. (2003). MINITAB statistical software, release 14 for windows. Minitab Inc., State College, USA.

Nawab NN, Subhani GM, Ullah MN **(**2013). Patterns of morphological diversity and character association in Chickpea genotypes through multivariate approach. The Journal of Animal & Plant Sciences, 23(4):1107-1114.

Nei M (1972). Genetic distance between populations. The American Naturalist 106(949):283-292.

Pavlicek A, Hrda S, Flegr J (1999). Short Communication FreeTree-Freeware Program for Construction of Phylogenetic Trees on the Basis of Distance Data and Bootstrap/Jackknife Analysis of the Tree Robustness. Application in the RAPD Analysis of Genus Frenkelia. Folia Biologica (Praba), 45:97-99.

Raval LJ, Dobariya KL (2003). Yield components in improvement of chickpea (*Cicer arietinum* L.). Annals of Agricultural Research, 24:789-794.

Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica, 128:9-17.

Rohlf FJ (2000). Numerical taxonomy and multivariate analysis system version 2.1. Exeter Publishing, New York.

Saleem M, Tahir M, Kabir R, Javid M, Shahzad K (2002). Interrelationships and path analysis of yield attributes in chickpea (*Cicer arietinum* L.). International Journal of Agriculture & Biology 3:404-406.

Tesfamichael SM, Githiri SM, Nyende AB, Rao NV (2015). Variation for Agro-Morphological Traits among Kabuli Chickpea (*Cicer arietinum* L.) Genotypes. Journal of Agricultural Science, 7(7):75-92.

Toker C (2004). Evaluation of yield criteria with phenotypic correlations and factor analysis in chickpea. Acta Agriculturae Scandinavica, Section B-Soil & Plant Science, 54(1):45-48.

Wallace DH, Yan W (1998). Plant breeding and whole-system crop physiology: improving crop maturity, adaptation and yield. CAB international. pp. 19-390.

Wang X (2011). Inter-simple sequence repeats (ISSR) molecular fingerprinting markers for authenticating the genuine species of rhubarb. Journal of Medicinal Plants Research, 5(5):758-764.

Yeh FC, Yang RC, Boyle T (1999). POPGENE version 1.31. Microsoft window-based freeware for population genetic analysis. University of Alberta, Edmonton, Canada.

Zali H, Farshadfar E, Sabaghpour SH (2011). Genetic variability and interrelationships among agronomic traits in chickpea (Cicer arietinum L.) genotypes. Crop Breeding Journal, 1(2):127-132.

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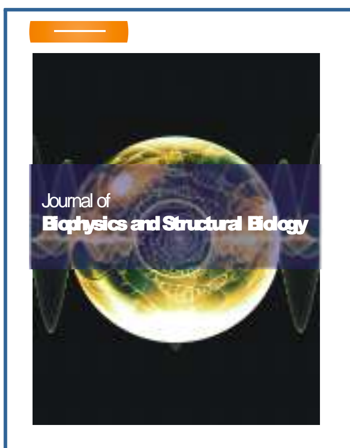

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