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Full Length Research Paper

Physiological performance of heat stressed growing rabbits fed diets supplemented with vitamin anti-oxidants and bicarbonate buffers

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The aim of the study was to evaluate the physiological performance of heat stressed growing rabbits fed diets supplemented with vitamin anti-oxidants and bicarbonate buffers. A total of thirty (30) growing rabbits (New Zealand White crosses) of two months old were used in this study. There were six (6) rabbits per treatment in a Completely Randomized Design (CRD). The treatment groups consisted of: control, sodium bicarbonate ($NaHCO_3$) and potassium bicarbonate, ($KHNO_3$) solution respectively, Vitamin C, and baobab fruit pulp meal (BFPM) as supplements, respectively. The experiment lasted for 9 weeks. Five milliliters of blood were collected from five rabbits (through the ear vein) chosen randomly from each group of rabbits, respectively at the beginning and the end of the experiment for serum metabolite and thyroxine hormone evaluation. Physiological performance of the rabbits was also evaluated. It was found that environmental conditions were stressful to the animals; Vitamins (Vit C and BFPM) significantly ($P<0.05$) reduced rectal temperature, heart rate and triglycerides compared to other treatments. The buffers recorded significantly ($P<0.05$) high feed intake, calcium and thyroxine. It was concluded that ameliorating heat stress with the antioxidants were helpful to improve the performance of rabbits and was recommended to be included in rabbit diets during the hot period.

Key words: Antioxidants, buffers, heat stress, thyroxine.

INTRODUCTION

Rabbits have been identified as a micro livestock with high economic potentials that can be used to bridge the gap for dietary protein intake in Nigeria. Rabbit meat poses a lot of health benefits by supplying micro nutrients which are deficient in leguminous grains and it is also low in cholesterol. Rabbit as a micro livestock can play a vital role in solving the world problem of deficiency of animal proteins (Daader et al., 2016) because of their high-quality protein, short generation interval, prolificacy and fast growth rate (Lebas et al., 1986). Improvement and

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increasing rabbit population have a significant role in the mitigation of poverty in the developing countries, where it is raised. Rabbit enterprise has also a main role in employment of rural communities' population.

Heat stress has been a major challenge in tropical and subtropical countries on rabbit production leading to impairment of both productive and reproductive performance (Marai et al., 2004, 2001). During stress, the endocrine system suffers (Chauhan et al., 2014). Adrenal corticosteroids are secreted during heat stress. High level glucocorticoids accelerate the metabolic rates and increase free radicals especially reactive oxygen species (ROS) (Ross et al., 1985; Sivakumar et al., 2010; Chauhan et al., 2014). Although low levels of ROS are essential for many biochemical processes, their accumulation due to over-production or a decreased antioxidant defense, leads to damage of biological macromolecules and disruption of normal cell metabolism (Spurlock and Savage, 1993). During heat stress, the few antioxidants in the body are depleted and induce oxidative stress (Abou-Ashour et al., 2004).

Vitamin C (ascorbic acid) and E are vitamin antioxidants that have been widely used to alleviate heat stress in rabbits and poultry (Yassein, 2010; Arafa Mervat et al., 2012). Vitamin C is prominent in the defense against superoxide ions, singlet oxygen and other free radicals. It also neutralizes RNA. Ascobic acid has the potential of dissolving in blood and cytosol and can act quickly before cell damage occurs. Plants and their parts could serve as a phytobiotics and antioxidants to the livestock (Dhama et al., 2015; Valenzuela-Grijalva et al., 2017) because of the presence of vitamin C and other phytochemicals in them. Some phytochemicals in plants improve antioxidant, anti-microbial, feed flavour and palatability which could result in increased feed intake and performance in animals (Valenzuela-Grijalva et al., 2017). These tropical plants are available because of their rapid growth which is enhanced by the prevailing and environmental factors. Baobab has been described by Williams (2002) and Phyto Trade Africa (2009). The fruit pulp was reported to contain high amounts of vitamin C (Sena, 1998), it was reported to increase feed intake, weight gain and was effective in alleviating heat stress in rabbits (Anoh, 2017). $NaHCO_3$ was found to improve oxidative stress and heat tolerance by immune-modulation. Sodium bicarbonate in feed or water was reported to improve growth performance (Ahmad et al., 2005; Khattak et al., 2012; Peng et al., 2013), egg quality (Kaya et al., 2004; Jiang et al., 2015) and improve blood profile (Kurtoglu et al., 2007) in poultry birds and rabbits that were affected by heat stress. The chemical is cheap, easily available and easy to handle, therefore, can be safely used to ameliorate the adverse effects of heat stress.

This study was designed to evaluate effect of vitamin anti-oxidants and bicarbonate buffers on physiological performance and thyroxine secretion of heat stressed growing rabbits.

MATERIALS AND METHODS

Experimental site

This study was carried out at the Rabbit Unit of the National Animal Production Research Institute (NAPRI) Shika, Zaria. Shika lies between 11° 12' 42" N and 7° 33' 14" E at an altitude of 691 m above sea level (Ovimaps, 2014). Zaria has an average rainfall of 1100 mm which starts from late April and early May to mid-October and an average temperature of 37°C and average relative humidity of 75%.

Preparation of buffer

Potassium bicarbonate, sodium bicarbonate and carbonate anhydrous salts were purchased from a laboratory equipment and chemicals vendor in Samaru-Zaria Nigeria. Distilled water was prepared in the Multiuser Laboratory of the Department of Chemistry, Ahmadu Bello University, Zaria. The buffer solution was prepared according to the methods of Chandra (2006) at a pH of 7.5 in the Department of Biochemistry, Ahmadu Bello University, Zaria.

Experimental animals, diets and design

A total of thirty (30) growing rabbits (New Zealand White crosses) of two months old were used in this study. There were six (6) rabbits per treatment in a Completely Randomized Design (CRD). The treatment groups consisted of: control, sodium bicarbonate ($NaHCO_3$) and potassium bicarbonate, ($KHNO_3$) solution, respectively, Vitamin C, and baobab fruit pulp meal (BFPM) as supplements, respectively. The basal diet composition (kg) was as follows: maize 30, groundnut haulm 20, groundnut cake 10, soybean meal 15, rice bran 15, bone meal 9.2, common salt 0.35, mineral-vitamin premix 0.25, dl-methionine 0.1 and lysine 0.1. The basal diet was formulated to meet the nutrient requirements of growing rabbits according to the recommendations of NRC (1994). The calculated analysis of the nutrient composition of the basal diet is shown in Table 1. The buffer solution was offered *ad libitum* but changed daily in the morning. All recommended managerial practices were duly observed and the study lasted for 9 weeks.

Housing

The animals were housed in perforated metallic hutches measuring 75 × 75 × 75 cm and raised 80 cm from the floor level in a naturally ventilated building. The hutches were thoroughly washed and disinfected with a locally made disinfectant and allowed to dry for one week before the animals were brought. Feed and watering troughs which were made of burnt clay were provided in each hutch. The rabbits were placed individually in clearly labeled cells.

Meteorological data of rabbit microclimate

The microclimate (ambient temperature and relative humidity values) within the rabbit house was recorded twice daily at 08:00 and 15.00 h during the study period using a digital thermometer

Table 1. Calculated composition of the experimental diets.

Nutrients	Composition
Metabolizable energy (kcal/kg)	2200
Crude protein (%)	18
Crude fiber (%)	10

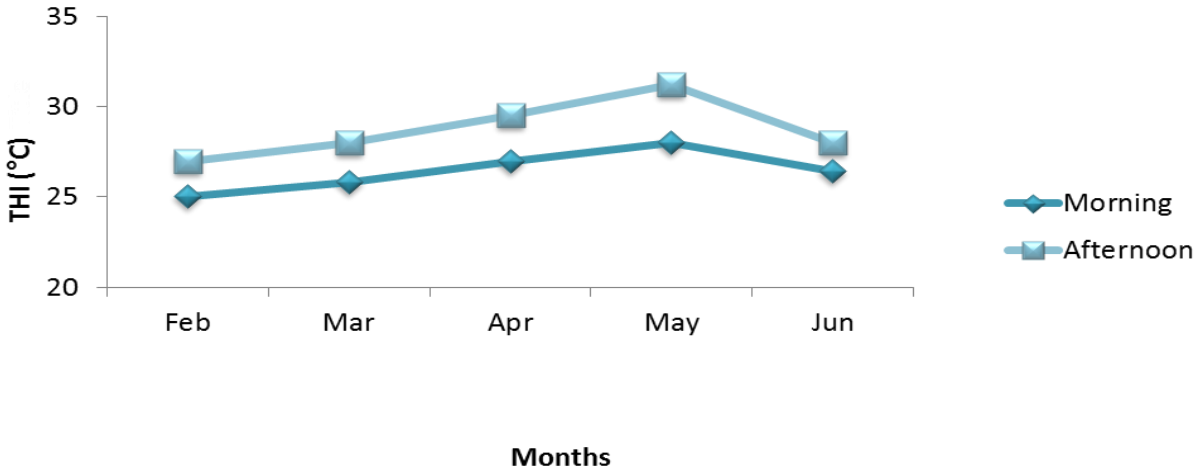

Figure 1. Monthly temperature humidity index of the pen house.

(Cocet, Shenzhen-Guangdong, China). The data collected was used to compute the temperature-humidity index (THI), an indicator of the thermal comfort level of the rabbits. The THI was calculated using the modified formula for the rabbit by Marai et al. (2001) as follows:

$$THI = t - [(0.31 – 0.31 \times RH) (t – 14.4)]$$

where RH = relative humidity /100 and t = ambient temperature.

The values of THI obtained were compared to that classified for tropical regions as follows:

1) <27.8 = Absence of heat stress, 2). 27.8 - 28.9 = Moderate heat stress, 3) 28.9 – 30 = Severe heat stress, and 4) above 30 = Very severe heat stress.

Physiological performance evaluation

Measurements of rectal temperature (RT) and heart rate (HR) were taken at 14.00 to 15.00 h of the day. Rectal temperature was measured with a digital thermometer and HR was measured by counting the heartbeat of each rabbit representing their treatment for 1 min with the help of a stethoscope. Weight gain and feed intake was determined weekly.

Serum evaluation

Blood sampling was done every two weeks at 10.00 h. Four rabbits were randomly selected from each treatment group and 5 ml of blood was collected from their ear veins into sample bottles without anticoagulants. The blood sample was allowed to clot and the serum was harvested after centrifuging the samples at 3000 rounds/min for 15 min. The serum harvested was stored at -10°C until when analyzed. Serum thyroxine concentrations were determined by using commercially available ELISA Kits (Diagnostic Procedure Corp., Los Angeles, CA, USA) according to the manufacturer's instructions. Serum glucose, total protein, albumin, and cholesterol concentration were evaluated using an auto-analyzer and Chemical Commercial Kits from Stanbio Laboratory Inc. San Antonio, Texas, USA, according to the manufacturer's instruction. The detection range of the T_4 was 26 to 58.4 g/mg.

Statistical analysis

Data obtained from the study were subjected to analysis of variance using the general linear model procedure of SAS (2002). Significant differences among treatment means were separated using the pairwise difference (Pdiff) in the SAS package.

RESULTS

Monthly temperature-humidity index (THI)

The monthly temperature-humidity index (THI) inside the rabbitry during the experimental period is as shown in Figure 1. THI in the mornings averaged 26.44°C while the afternoon THI averaged 28.74°C. This graph also shows that the THI values kept increasing from the month of

Table 2. Effects of vitamin anti-oxidants and bicarbonate buffers on physiological performance of growing rabbits treatments.

Parameter	Control	$KHCO_3$	$NaHCO_3$	Vit. C	BFPM	SEM
Rectal temperature (^oC)	37.68^a	36.56^{bc}	37.27^a	35.87^c	35.73^c	0.31
Heart rate (beats/min)	141.03^b	143.36^a	143.19^a	141.35^b	141.29^b	0.53
Feed intake (g/day)	30.76^b	30.93^b	42.39^a	37.59^a	36.77^a	1.88
Weight gain (g/day)	13.96	16.99	14.58	13.37	15.28	1.82

Means within rows with different superscripts are significantly different: p<=0.05, SEM= Standard Error of Mean, Vit. C = Vitamin C, BFPM = Baobab fruit pulp meal

Table 3. Effects of vitamin anti-oxidants and bicarbonate buffers on serum metabolites of growing rabbits treatments.

Parameter	Control	$KHCO_3$	$NaHCO_3$	Vit. C	BFPM	SEM
Glucose (mg/dl)	3.50	3.30	3.60	4.80	5.00	0.12
Total Protein (mg/dl)	66.67	65.67	72.00	69.67	72.67	0.38
Albumin (mg/dl)	34.67	36.00	37.67	37.33	38.67	1.38
Cholesterol (mg/dl)	1.33	1.30	1.33	1.43	1.43	0.04
Triglyceride (mg/dl)	0.97^b	1.00^{ab}	1.00^{ab}	1.30^{ab}	1.40^a	0.08
Calcium (mg/dl)	2.39^{ab}	2.31^{ab}	2.30^{ab}	2.33^a	2.26^b	0.01
Phosphorous (mg/dl)	1.08	1.13	1.31	1.16	1.09	0.01

Means within rows with different superscripts are significantly different: p<=0.05, SEM= Standard Error of Mean, Vit. C = Vitamin C, BFPM = Baobab fruit pulp meal.

February with a peak in May. There was a decline in THI in the month of June.

Physiological evaluation

Vitamins (Vit C and BFPM) (Table 2) significantly ($p<0.05$) reduced rectal temperature and heart rate compared to other treatments. The range values were 37.68 (control) to 35.73 (BFPM). Feed intake significantly ($p<0.05$) increased in the treatments with $NaHCO_3$, Vitamin C, and BFPM (42.39, 37.59, and 36.77), respectively compared to $KHCO_3$ (30.93) and the control (30.76).

Serum metabolite evaluation

There was a significant ($p<0.05$) increase in the values of triglycerides (Table 3) for the Vitamins (1.30 and 1.40) compared to the buffers (1.00) and the control (0.9). Vitamin C recorded significantly ($p<0.05$) higher calcium compared to BFPM which was the lowest among the treatments.

Thyroxine secretion evaluation

The result in Figure 2, shows that Initial thyroxine levels were generally low compared to final thyroxine secretions. The trend in the values of the final thyroxine secretions was a reflection of the trend in the thyroxine of the initial secretions. The buffers recorded significantly ($P<0.05$) higher thyroxine levels (67.12 and 66.92) compared to treatments with vitamins (65.45 and 66.10). Going by the difference in initial vs final thyroxine secretion (3.46 vs 3.60) of $NaHCO_3$ and BFPM, respectively, it was observed that the difference in the increase was higher for BFPM treated rabbits than $NaHCO_3$ treatment.

DISCUSSION

The THI value of 27°C (February) indicated that the month of February had absence of heat stress in the rabbit house, while the THI values of 28°C (March), 29.5°C (April), 31.2°C (May) and 28°C (June) are indications that the rabbit house was moderately thermally stressful, severely thermally stressful, and very severely thermally stressful (Marai et al., 2001) in these months. The averaged THI 28.74°C during the experimental period indicated that the rabbit house was thermally stressful and may have had adverse effects on the rabbits (Marai et al., 2001). Overall data obtained indicated that THI in the afternoon was higher by 1.24% than THI in the morning.

The increase in feed intake in $KHCO_3$ treatment agrees

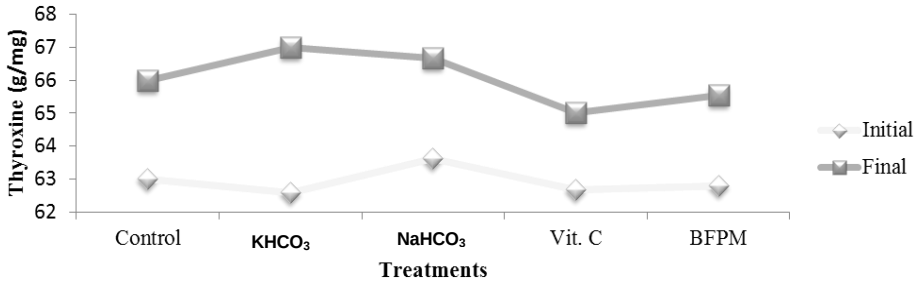

Figure 2. Effects of vitamin anti-oxidants and bicarbonate buffers on thyroxine levels in growing rabbits.

with the findings of Yassein et al. (2011) who reported that $NaHCO_3$ and $KHCO_3$ can serve as appetizer supplement to rabbits diets which may stimulate the appetite, increase fiber digestibility and improve feed efficiency (Abdel-Samee et al., 2003). Sodium bicarbonate in feed or water has shown potential benefits on production performance (Ahmad et al., 2005; Khattak et al., 2012; Peng et al., 2013), egg characteristics (Kaya et al., 2004; Jiang et al., 2015) and blood profile (Kurtoglu et al., 2007) in poultry birds and rabbits exposed to heat stress.

Marai and Habeeb (1994) found that 1.25 or 2.5% $NaHCO_3$ improved growth performance, rectal temperature, respiration rate and blood components due to correcting acid-base balance disturbances, such as under stress conditions. Vitamin C has been reported to be effective in the growth performance of rabbits especially during heat stress (Rao and Sharma, 2001). Antioxidant vitamins were effective to alleviate heat load in rabbits (In-Surk et al., 2014; Prabsattroo et al., 2012). The administration of ascorbic acid (Mckeeand and Harrison, 2013), glutathione supplementation (Sahin et al., 2003), during exposure to high environmental temperatures reduces the body temperature in chickens. In heat-stressed sheep, selenium injection decreased rectal temperature and body weight loss (Alhidary et al., 2012). The presence of the phytochemical compounds in plants, vitamins may facilitate the ability of animals to maintain their body homeostasis including body temperature by provoking endogenous cellular defense mechanisms to cope with oxidative stress and inflammation induced by heat stress (Akbarian et al., 2016).

The values in final serum metabolites in this experiment followed the trend of the rabbit's growth performance records (feed intake, weight gain and final body weight) in this study. For instance, BFPM recorded a significantly high serum triglyceride which was similar to the treatments with vitamin C and bi-carbonate buffer compared to the control. It should be recalled that these treatments were the ones that showed a better feed intake and weight gain compared to the control. Triglycerides are an important component of the body's adipose tissue. Ambient temperature-induced heat stress was shown to reduce fat oxidation in different species; rodents (Sanders et al., 2009), pigs (Pearce et al., 2011) and dairy cows (Shwartz et al., 2009). Moreover, heat stress down-regulates lipolytic enzyme activities, as seen in chickens and swine (Geraert et al., 1996). Lebas et al. (1986), Chiericato et al. (1996) and Marai et al. (2001) reported a reduction in live body weight and daily body gain weight due to heat-stress conditions and were attributed to the negative effects of heat-stress on appetite and consequent decrease in feed consumption. Low feed intake might have affected the low serum metabolite noticed in the control group. The blunted lipolytic activity of the adipose tissue seems to be an adaptation form to limit heat generation in heat-stressed animals.

The marked differences in initial and final thyroxine levels can be attributed to changes in age and body metabolism of the rabbits. It should be known that body metabolism increases with age; T_4 have been reported to increase with increasing age in chicks (Leenstra et al., 1991). In the present study the buffers recorded a high thyroxine secretion; the buffer might have triggered the activity of the thyroid gland to increase thyroxine secretion. On the reduction in T_4 at the final stage by the treatments with vitamin C and BFPM meal diets agrees with the fact that thyroid the hormone increases the quantities of many bodily enzymes and because vitamins are essential parts of some of the enzymes or coenzymes, thyroid hormone causes increased need for vitamins (Guyton and Hall, 2006). Therefore, a relative vitamin deficiency can occur when excess thyroid hormone is secreted, this will lead to a consequential depletion and the need for more vitamins to maintain homeostasis and will, in turn, reduce thyroxine secretion, unless at the same time increased quantities of vitamins are made available. Serum concentrations of T4 increased by increasing dietary Vitamin C or Vitamin E levels of heat-stressed Japanese quails (Sahin et al.,

2002) and rabbits (Daader et al., 2018). Thyroid hormones are the key hormones in the regulation of metabolism and adaptation of animals to stress (Brecchia et al., 2010).

Conclusion

Ameliorating heat stress with the antioxidants was helpful to improve the performance of rabbits. Vitamin antioxidants performed better than the bicarbonate buffers and were recommended to be included in rabbit diets during the hot period.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Efficacy of Sudanese isolates of entomopathogenic fungi against the Khapra beetle *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae)

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The current study evaluated the bio-control activity of Sudanese isolates of entomopathogenic-fungi against 3rd larval instars of Khapra beetle (*Trogoderma granarium*) (Everts) in Sudan and morphologically and molecularly characterized the virulent isolates. Fungi were isolated using Galleria-baiting method and tested against the larvae using immersion-technique at concentration of 1×10^7 conidia ml^{-1}. Commercial product, Eco-Bb® was used as standard treatment. Twenty of the 3rd instar larvae were immersed in 10 ml of fungal suspension for five seconds. Control larvae were immersed in sterilized-distilled water. Dead insects were counted daily for seven days after inoculation. Microscopic examination of the cadavers was conducted to explain whether or not the test organisms caused the death of test larvae. Virulent isolates were identified morphologically and confirmed by molecular techniques as *Beauveria bassiana* isolate Sud-afro.18 (MK046654), *Metarhizium anisopliae* isolate Dmazeen F1 (MK046658), *Metarhizium anisopliae* isolate Dmazeen R1 (MK046659), *Albifimbria viridis* isolate Shmbat-fo1 (MK046656), *Purpureocillium lilacinum* isolate Khartoum f1 (MK046655), *B. bassiana* isolate Sud-afro.20 (MK046652), *B. bassiana* isolate Giddo6RF (MN598664), *B. bassiana* isolate GiddoR (MN598665), and *B. bassiana* isolate HammadR7,F (MN598666). Mortality induced by various isolates ranged from 40.0-90.4% compared to 96 and 7.9% induced by standard treatment Eco-Bb® and untreated control respectively. LT_{50} values of *B. bassiana* isolate Sudafro.18 and *M. anisopliae* isolate DmazeenF1 is comparable to that induced by Eco-Bb®.

Key words: Entomopathogenic fungi, identification, biological control, Khapra beetle, Sudan.

INTRODUCTION

Sorghum, *Sorghum bicolor* (L.), is an important cereal crop grown worldwide. It is a member of the family

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Gramineae and used for food and feed purposes. It is mostly cultivated in the semi-arid tropics where water is scarce and drought is frequent (Bashir, 2011). Sorghum ranks second among cereals and the fifth among all crops in terms of production in Africa. It is the most important food crop in Sudan, where it is grown on both rain-fed and irrigated commercial farms. Sudan contributes with 6.51 to 9.6% of global sorghum production. However, sorghum production in Sudan suffered from the attack of insects, weeds, diseases, birds, rodents, and other pests. In storage, sorghum grain is attacked by many pests, especially the members of Coleoptera and Lepidoptera, which cause serious losses in both quantity and quality of sorghum grain and other stored products. The Khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera Dermestidae), and other beetles of the Dermestidae are considered to be the most destructive pests of stored products (Burges, 2008; Mark et al., 2010) globally and in Sudan. The pest was first reported in Sudan in 1944 and has established itself as the most important storage pest in the driest regions of Sudan (Saad, 1969). The insect deposits its eggs usually on the surface of the grain. The larvae hatch, penetrate the grain, feed, and develop. In this process, the larvae contaminate the grain in storage with frass and detached body parts. Severe infestations can make the grain unpalatable, and therefore unmarketable (Anonymous, 1982; Mason, 2002).

Globally and in Sudan, synthetic pesticides have been used to control stored grain insects, including the insecticides lindane, malathion, chlorpyrifos-methyl, bioresmethrin, resmethrin, deltamethrin, and fumigants such as phosphine and methyl bromide. The latter was listed as a category 1 ozone-depleting substance under the Montreal Protocol 1992 and therefore was banned globally (Arthur, 2000; UNEP, 2016). Furthermore, most storage pests have developed resistance to many of the widely used insecticides (Ecresam, 2005; Rossi et al., 2010). The biological control of stored-grain insects with natural enemies and pathogens, alone or in combination with selective insecticides, has been proposed as an alternative to non-selective insecticides and fumigants (Thompson and Brandenburg, 2006). Edde (2012) claimed that biocontrol agents are safe for human health, do not pollute the environment, and do not accumulate in the eco-systems in which they are used. Most microbial biopesticides used are living organisms, which are pathogenic to the pest with a very high safety margin to human health and non-target organisms and are largely harmless to beneficial organisms (non-target species), including warm-blooded animals and humans (Canan, 2013; Medeiros et al., 2018; Mantzoukas and Eliopoulos, 2020). Entomopathogenic fungi play vital role as biocontrol agent of insect populations and therefore have been used in many successful integrated pest management programs (Cana, 2013; Wakil et al., 2014; Kaushal et al., 2016; Khun et al., 2020; Deka et al., 2021). Given their relative safety, the fungal biocontrol agents could be used to protect foods and feeds from specialized storage pests (Cox et al., 2016). Previous studies reported that *Purpureocillium lilacinum*, *Beauveria bassiana*, *Metarhizium anisopliae* Metchn (V275 & V245), and *Verticillium lecanii* Zimm (KV183) are effective biological control agents against *Tuta absoluta*, *Sitophilus zeamais* Motsch and *Sitophilus oryzae* (Buba, 2010; Kavallieratos et al., 2014; Hammad et al., 2021). In another work, *Isaria fumosorosea* and *M. anisopliae* were reported as effective against adults of the stored-grain insect pests *Sitophilus granaries*, while *M. anisopliae* was found effective against *S. oryzae* (Ak, 2019). In another study, Wakil et al. (2014) found that the cadavers of red flour beetle *Tribolium castaneum* were found significantly infected with the fungi followed by rice weevil *S. oryzae* (L), lesser grain borer *Rhyzopertha dominica* (F.), rusty grain beetle *Cryptolestes ferrugineus* (Stephens), and cowpea weevil *Callosobruchus maculatus* (F.), while the least infection were recovered from Khapra beetle *T. granarium* (Everts). Other than evaluation of some plant extracts, Yousif and Satti (2012), Abdalla and Abdelbagi (2015a, b), Mahmoud et al. (2015), Sir El Khatim and Abdelbagi (2015), Abdelbagi et al. (2018), Sir El Khatim et al. (2018), Ahmed et al. (2019), and Ishag et al. (2019) noted that no prior research has been undertaken in this field in Sudan despite the numerous studies conducted on this topic worldwide. Therefore, the main objective of this study was to characterize and evaluate the efficacy of Sudanese isolates of entomopathogenic fungi against the Khapra beetle, *T. granarium* (Everts) (Coleoptera: Dermestidae).

MATERIALS AND METHODS

Rearing of the Khapra beetle *Trogoderma granarium*

Sound sorghum grain was sterilized by heating to 120°C for fifteen minutes in an oven to eliminate any pests present in the grain (Winks, 1982). The sterilized grain was taken out and stored at room temperature for experimental purposes.

Adults of the Khapra beetle were obtained from infested sorghum grain obtained from the local markets, sieved, and reared in three glass jars (3 kg capacity) half-filled with crushed sorghum grains. The jars were then covered with a muslin cloth fixed in position with a rubber band and kept in the laboratory at room temperature. Adults of Khapra beetle were allowed to lay their eggs on crushed sorghum grains at optimum conditions (35°C, 65% RH). After three months of rearing, the 3rd instar larvae of the third generation were collected and used for further experiments.

Entomopathogenic fungal isolates

A commercial product of *B. bassiana* Strain R444 (Eco- Bb®,) was imported from Plant Health Product (Pty) Ltd, South Africa and was used as a standard biopesticide against which the performance of some local strains of entomopathogenic fungi was compared. The

Eco-Bb® product was selected as standard based on its reported potential against a wide range of agricultural pests including whiteflies, red spider mites, *T. absoluta,* and false codling moth (Hammad et al., 2021). Although none of the previous studies used this product against the test pest, its efficacy against it is expected. Isolation of the endogenous isolates was done by two methods.

Selective media method

The selective media was made by 16.25 g of potato dextrose agar (PDA Merck) with one ml of Dodine (used to reduce the microbial contaminants), and 250 ml of distilled water in a conical flask, which was then autoclaved for 20 min. The medium was allowed to cool at room temperature (30°C), thereafter 500 µl of chloramphenicol and 500 µl of streptomycin sulphate were added to the flask (Strasser et al., 1996). The contents of the conical flask were shaken gently by hand to mix evenly and then poured into plastic Petri dishes on a laminar flow bench.

Precisely 100 g of soil sample was taken and placed in a glass bottle containing 250 ml of distilled water, which was then sealed. The bottle was agitated vigorously for three hours using a digital reciprocating shaker (150 rpm) (WiseShaker, Wisd Laboratory Equipment, Daihan Scientific, Korea). The bottle was left for 24 h to allow the soil particle to settle down, and then a 100 µl of suspension was spread-plated on selective potato dextrose agar (sPDA), which was then sealed and incubated at 25°C. The observation of fungus growth continued from three to seven days of incubation. The white mycelium and conidia was observed and re-cultured several times in the selective medium to obtain a pure culture. The pure culture was then kept in an incubator for use in the experiment (Senanayake et al., 2020).

Insect baits isolation method

Rearing was performed according to the protocol described by Zimmermann (1986) and Meyling (2007) with some modifications. Galleria infested bee wax was collected from the Botanical Garden, University of Khartoum, placed in rearing containers under laboratory conditions (25°C and 65% RH) for four weeks, until the eggs hatched. The hatched larvae were collected for experimental purposes.

Five hundred milliliters of water were placed in a beaker placed in a water bath at 56°C; thereafter 10 of the larvae were removed from the rearing containers (containing moist soil as medium) placed in a mesh bag. Prevention of webbing the bagged larvae was done by 10 s dipping into the beaker of warm water followed by cooling in running water for 30 s. The larvae were then placed on dry tissue paper and kept in the dark for three hours after which the larvae had started to move and then placed in soil bottles, which were kept in an incubator at 25°C for 14 days, or until they died. Dead Larvae were rather hard: mummified and dark brown in colour. The bottles were monitored regularly for the dead larvae, which were collected and surface-sterilized with 1% sodium hypochlorite solution for three minutes, followed by gentle rinsing (2 times) with sterilized distilled water. Dead larvae were placed on a Petri dish of selective agar, incubated at 25°C and 65% RH in a growth chamber, and observed for the presence of fungal growth. Entomopathogenic fungi emerging from the cadavers were re-cultured several times to obtain pure cultures and maintained on PDA for subsequent experimental purposes.

Preparations of fungal suspensions

The method described by Bashir (2017) was followed in the preparation of fungal suspensions. Eco Bb® commercial product (0.05 g) and 100 µl of each local isolate culture were taken and suspended in a beaker (50 ml volume) containing 10 ml of sterile distilled water and 100 µl of Tween 20, and the suspension was agitated vigorously. A 100 µl aliquot of the conidial suspension was applied to a series of PDA plates and incubated at 25°C. After seven days of incubation, the fungal growth covered all the plates; thereafter the plates were left for 14 days to complete sporulation. The presence of spores was checked at the end of the sporulation period (21 days). Spore concentrations of suspensions were measured using hemocytometer slide. The fungal suspension (1×10^7 conidia ml) was prepared by adding 10 ml of sterile distilled water and 100 µl of Tween 20 to the Petri dishes containing the pure culture of each local isolate, and the standard kept for immediate use in the bioassay test.

Bioassays

The 3rd instars larvae of Khapra beetle were collected from the rearing jars and used to evaluate the efficacy of the isolated entomopathogenic fungi. Twenty larvae were taken, placed in Petri dish and immersed separately for five seconds in each of the fungal suspensions of the commercial product Eco-Bb® and the local isolates at a concentration of 1×10^7 spores ml^{-1} under laminar controlled conditions. The treated larvae were then placed in sterile Petri dishes containing moist filter papers fed with cooked sorghum grains, and maintained under laboratory conditions (25°C and 65% RH) until the end of the bioassay. The experimental units were arranged in a completely randomized design with four replicates. Mortality was recorded daily until the seventh day after inoculation where the experiments were terminated as pre-test, indicating that test insects can reach 100% mortality within seven days.

Mycosis test

A mycosis test was done following the method described by Akutse et al. (2013), Muvea et al. (2014), and Agbessenou et al. (2020) to confirm that test isolates are the causal agents for the death of treated insects as confirmed by the isolation of the inoculated isolates from the cadaver of death larvae.

Characterization of virulent isolates

Morphological characterization

Morphological identification of the entomopathogenic fungal isolates was done under a microscope following the morphological identification keys described by Rehner et al. (2011). The observations made on the colony characters included; the growth pattern, color, surface texture, and others (Hammad et al., 2021).

Molecular characterization

DNA extraction of virulent isolates: The nine most effective isolates were subject to molecular characterization. Seven days old fresh mycelium was ground and homogenized in liquid N_2, then 300 ml of DNA extraction buffer (200 mM Tris-HCL (PH 8.5) + 250 mM NaCl + 25 mM EDTA + 0.5% SDS {sodium dodecyl sulfate}) was added. The suspension was transferred to 1.5-ml Eppendorf tubes and the tubes were then placed in a water bath at 60°C for 60 min. 150 µL of 3 M sodium acetate (pH 5.2) was added and the mixture was cooled to 20°C for 10 min. The fungal debris was pelleted by

centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to a clean tube, and an equal volume of absolute ethanol (100%) was added. DNA was then pelleted by centrifugation at 13,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, thereafter the DNA pellets were re-suspended in 100 µL distilled water and stored at -20°C for PCR analysis.

Measuring DNA quality and quantity: The concentration and quality of DNA were measured using a nanodrop spectrophotometer (ND1000 Spectrophotometer, Nanodrop Technologies). The method was based on measuring the amount of ultraviolet (UV) radiation absorbed by the bases. The ratio of OD 260/280 was determined to assess the purity of samples. The DNA concentration was measured as ng μl^{-1} and was also checked by visualization on an agarose gel.

PCR amplification

Isolate genotype was amplified by the polymerase chain reaction (PCR) using a thermo cycler machine. An internal transcribed spacer (ITS) primer, forward ITS1 [TCCGTAGGTGAACCTGCGG] and reverse ITS4 [TCCTCCGCTTATTGATATGC], were used to amplify bases of about 570bp (White et al., 1990). Amplification reactions were done in a total volume of 25 µL containing a PCR ready mix kit (i-Taq), 2 µL of DNA template, 1 µL of forward primer, 1 µL of reverse primer, and distilled water. The reaction conditions were as follows: initial denaturation at 94ºC for 2 min, followed by 40 cycles, which consisted of the second denaturation at 94ºC for 45 s, 50ºC for 45 s for annealing, and 72ºC for 1 min for extension. The cycle was ended by a final extension at 72ºC for 7 min followed by 4ºC for 5 min.

Agarose gel electrophoresis

PCR products were separated by electrophoresis on 1.5% agarose gel. Ethidium bromide gel staining was applied, and the DNA fragments were observed under (UV) light and photographed. The molecular sizes of the fragments were estimated by comparison with a100bp DNA ladder.

Sequencing of the fungal ribosomal genes

All samples were sequenced by the use of the Sanger dideoxy sequencing method using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems), and a similarity search was performed using a Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database. Species confirmation was performed using the existing available database. Obtained sequences were submitted to the NCBI database and accession numbers were obtained.

Phylogenetic analysis

The phylogenetic trees were constructed using the neighbor-joining algorithm with Mega 6.0. The strengths of internal branches of the resulting trees were statistically evaluated by bootstrap analysis with 1000 bootstrap replications. The results of ITS rDNA sequence analysis were used to reconfirm the identity of the fungal isolates.

Statistical analysis

The collected data were subject to Arcsine transformation before the analysis. The experiment was arranged in a completely randomized design. The data were statistically analyzed by Analysis of Variance (ANOVA) according to Gomez and Gomez (1984). Means were separated using the least significant difference (LSD) test. Probit analysis (Finney, 1971) was done using MINITAB software version 13.20. Control mortality was corrected using Abbott's correction formula (Abbott's, 1925).

RESULTS

Morphological identification of entomopathogenic fungal isolates

Nine isolates of entomopathogenic fungi were isolated from soil collected from different sites in Sudan by the enrichment culture techniques.

Morphological identification of the entomopathogenic fungal isolates was done under the microscope following the morphological identification keys described by Rehner et al. (2011). The distinctive characters of the fungal growth included; growth pattern, color, surface texture, and the time required to cover the Petri dish. In the beginning, all colonies were white, and then became yellowish white. The underside of the colonies was pale to yellowish-white. Hyphae were hyaline, smooth-walled, and septate. Conidiogenous cells were solitary but generally appear in dense clusters. The colonies grew in a dispersed pattern and were rarely concentric in all the isolates whereas Eco-Bb grew in dense colonies. The colony color of the isolates of *B. bassiana* was yellowish-white; Isolates *Albifimbria viridis* isolate-Shmbat-fo1 and *Purpureocillium lilacinum* isolate-Khartoumf1 was white. Isolates of *Metarhizium* species is in green color (Figure 1).

Molecular characterization of the fungal pathogens

The entomopathogenic fungi were identified as *B. bassiana* isolate Sud afro.20 accession number MK046652, *P. lilacinum* isolate Khartoumf1 with accession number MK046655, *B. bassiana* isolate Sud afro.18 accession number MK046654, *M. anisopliae sensulato* isolate Dmazeen F1 accession number MK046658, *M. anisopliae sensulato* isolate Dmazeen R1 accession number MK046659 (Bischoff et al., 2009), *A. viridis* isolate Shambat fo1 accession number MK046656, *B*. *bassiana* isolate Giddo6 RF accession number MN598664, *B*. *bassiana* isolate Giddo R accession number MN598665, and *B*. *bassiana* isolate Hammad R7,F accession number MN598666 (Figure 2).

According to the phylogenetic analysis of the ITS gene region, the similarity among *B. bassiana* isolate Sud

Figure 1. Growth pattern and appearance of different isolates of indigenous entomopathogenic fungi (A: *B. bassiana* MK046652 B: *Metarhizium anisopliae* MK046658 C: *M. anisopliae* MK046659 D: *B. bassiana* MK046654 E: *B. bassiana* MN598664 F: *Albifimbria viridis* MK046656 G: *B. bassiana* MN598666 H: *Purpureocillium lilacinum* MK046655 I: *B. bassiana* MN598665 J: ECO- Bb444 product).

afro.18 (MK046654), *B. bassiana* isolate Giddo6 RF (MN598664), *B. bassiana* isolate Giddo R (MN598665), and *B*. *bassiana* isolate Hammad R7,F (MN598666) was 49%, while between the *B. bassiana* isolate Sud afro.20 *(*MK046652), *B. bassiana* isolate Sud afro.20 (MK046652) and other *Beauveria* isolates was 99%. On the other hand, 100% similarity was found between *M. anisopliae sensulato* isolate Dmazeen F1 (MK046658) and *M. anisopliae sensulato* isolate Dmazeen R1 (MK046659). While the *A. viridis* isolate Shmbat fo1 (MK046656) showed 91% similarity to *Metarhizium* isolates. In contrast, the *P. lilacinum* isolate Khartoumf1 MK046655 did not show any degree of similarity with any of the other isolates.

Efficacy of entomopathogenic fungi against the 3rd instar larvae of *Trogoderma granarium*

The mortality induced by the various isolates is given in Table 1. All treatments induced significant mortalities compared to the untreated control. The local isolate *B. bassiana* isolate Sud afro.18 (MK046654) induced comparable effects to the standard isolate Eco Bb® by day 7 although its effects were superior to it in day five and six; causing 87% and 90% in day 5 and 6 compared to 55%, and 71% induced by Eco Bb® during the same period (Table 1). *B. bassiana* isolate Giddo R (MN598665) and *B. bassiana* isolate Sud afro.20 (MK046652) came in the second rank inducing 80% mortality by day 7 while *B. bassiana* isolate Hammad R7,F MN598666 occupied the last rank with mortality not exceeding 40% even after day 7. Most of the other local isolates induced close to 70% mortality by day 7 (Table 1).

Mycosis test

The mycosis tests indicated that the test isolates were the causal agents of mortality among treated larvae. The fungal growth from cadavers appeared after 3-7 days of incubation at 25ºC and the dead larvae were completely covered with white fungal hyphae emerging from the larval cuticle. The dead larvae were soft, pliable, and distended. Within a few days after death, the larval body turned dark brown. Test isolates were recovered from the dead body of the treated larvae as confirmed by morphological and molecular techniques. No other pathogens were recovered from the dead larvae.

Time response of the 3rd larval instars of *T. granaium* to inoculation by local isolates of entomopathogenic fungi

The log time probit data of the 3rd larval instars of *T. granarium* dipped in the fungal suspension of various local isolates are shown in Table 2. Isolates gave LT_{50}

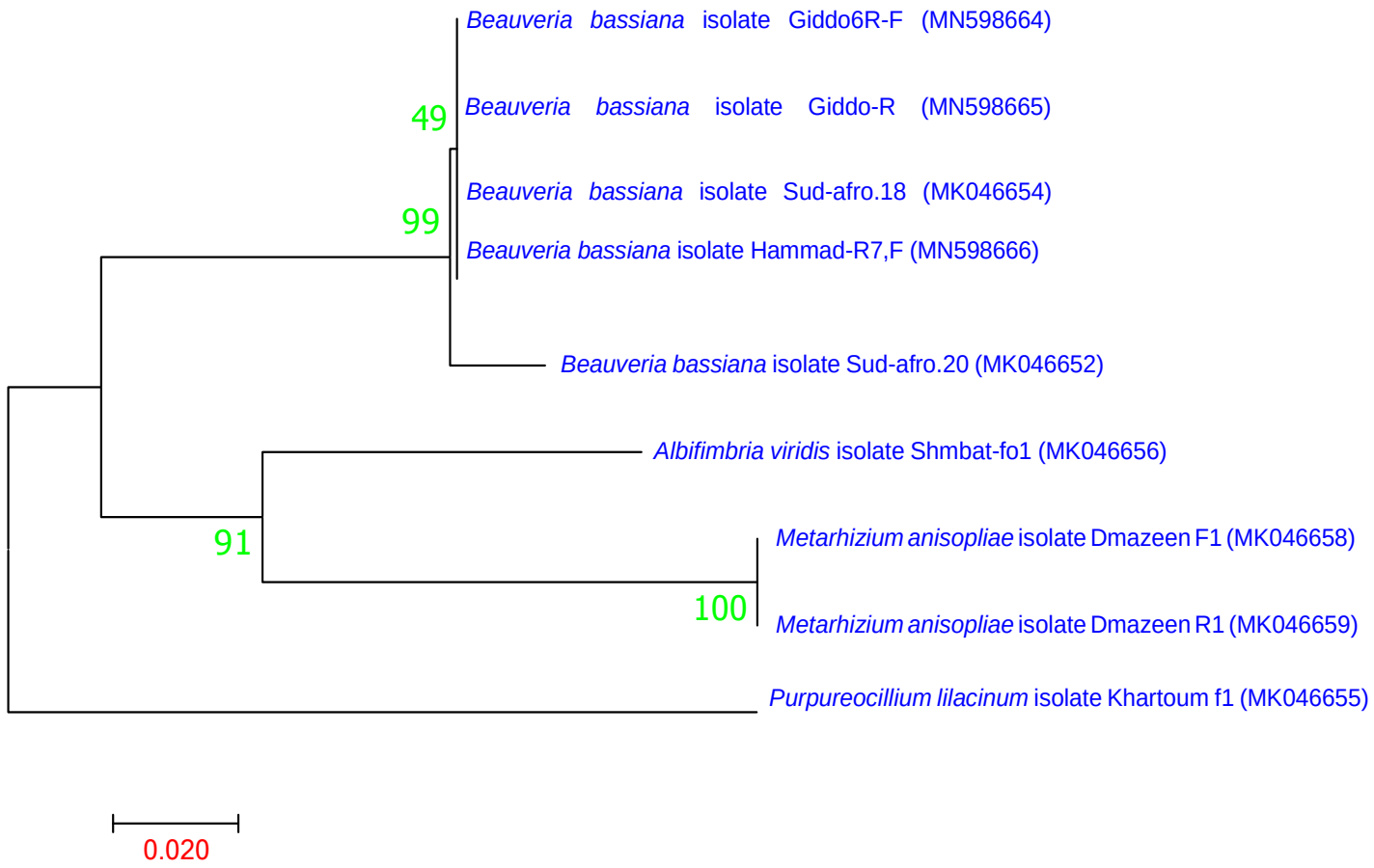

Figure 2. Neighbor-joining tree of indigenous isolates of entomopathogenic fungi (*Beauveria bassiana* MK046654, *Metarhizium anisopliae* MK046658, *M. anisopliae* MK046659, *Albifimbria viridis* MK046656, *Purpureocillium lilacinum* MK046655, *B. bassiana* MK046652, *B. bassiana* MN598664, *B. bassiana* MN598665, and *B. bassiana* MN598666) based on ITS gene region.

values ranging from 4.17-6.67 days. Based on the LT_{50} values, *M. anisopliae* isolate Dmazeen F1 (MK046658) ($LT_{50,}$ 4.23 days), and *B. bassiana* isolate Sud afro.18 (MK046654) ($LT_{50,}$ 4.27 days) gave the fastest response with LT_{50} close to the standard product Eco-Bb® (4.17 days). On the other hand, *M. anisopliae* isolate Dmazeen R1 (MK046659) ($LT_{50,}$ 6.67 days) and *B*. *bassiana* isolate Hammad R7,F (MN598666) (LT_{50} 6.60 days) gave the slowest response. The relative potencies followed the same trend. The LT_{90}/LT_{50} ratios were narrow ranging from 1.96 to 3.11 indicating fair homogeneity in the response of the test population. The similar homogeneity of response was also evident by the line slopes ranging from 0.22 - 0.38. The Chi-square value is small indicating good execution of the experiment (Table 2).

DISCUSSION

In this study, the pathogenicity of nine potentially effective isolates of local entomopathogenic fungi against the 3rd instar larvae of Khapra beetle *T. granarium* was assessed under laboratory conditions (25°C and 65% RH). Results indicated that the 3rd instar larvae of *T. granarium* were susceptible to the test isolates. The susceptibility of the 3rd larvae of *T. granarium* to the conidia or blasto-spores of different isolates of entomopathogenic fungi was previously reported by Whang et al., (2007) who obtained 100% mortality in 3rd larvae of *T. granarium*.

Most of the isolates were found effective against the test insect and three of them gave results comparable to the standard treatment, reaching 90.4, 80.4, and 80% mortality by day seven compared to 96% mortality caused by the commercial product Eco-Bb®. However, the effects induced by these three were even superior to the standard in day five and six. Variation in virulence among test strains were also reported by Zare et al. (2014) studying *M. anisopliae* (Iranian isolate) and *B. bassiana* (Bulgarian isolate) against *T. granarium* larvae. Their study showed that active larvae of *Trogoderma*

Table 1. Mortality of 3rd instar larvae of khapra beetle (*Trogoderma granarium)* after treatment with local isolates of entomopathogenic fungi and commercial product Eco Bb®.

Treatment	Mortality (%)						
	Days after Treatment						
	1	2	3	4	5	6	7
Eco Bb®	0.0^{A}	15.01^{A}	30.1^{A}	35.0^{B}	55.1^{E}	70.7^{C}	95.9^{A}
B. bassiana MN598664	1.1^{A}	5.1^{B}	30.1^{A}	35.1^{B}	45.1^{H}	45.1^{F}	54.0^{F}
A. viridis MK046656	3.3^{A}	5.1^{B}	15.0^{B}	25.4^{C}	59.5^{D}	59.5^{D}	74.6^{D}
B. bassiana MN598665	1.1^{A}	14.9^{A}	20.1^{C}	30.0^{C}	50.01^{F}	80.4^{B}	80.4^{C}
B. bassiana MN598666	5.1^{A}	15.01^{A}	15.01^{B}	20.1^{D}	40.0^{G}	40.0^{H}	40.0^{H}
B. bassiana MK046654	0.0^{A}	0.0^{C}	0.0^{D}	20.1^{D}	86.7^{A}	90.4^{A}	90.4^{B}
P. lilacinum MK046655	0.0^{A}	5.1^{B}	35.1^{E}	35.1^{B}	30.0^{N}	40.0^{H}	67.2^{E}
B. bassiana MK046652	1.1^{A}	10.0^{A}	35.1^{E}	35.1^{B}	40.0^{G}	51.3^{E}	79.7^{C}
M. anisopliae MK046658	5.1^{A}	15.01^{A}	49.1^{F}	71.5^{A}	72.6^{B}	72.6^{C}	72.6^{D}
M. anisopliae MK046659	0.0^{A}	10.0^{A}	10.0^{G}	35.1^{B}	61.8^{C}	61.8^{D}	73.8^{D}
Control	0.0^{A}	0.0^{C}	0.0^{H}	0.0^{E}	5.1^{A}	7.9^{G}	7.9^{G}
SE	3.24	3.14	2.18	3.20	2.26	2.51	2.58
LSD	5.49	5.32	3.70	5.43	3.84	4.24	4.37

Means with the same letter(s) in the same column are not significantly different at P = 0.05. Values represent the actual (not transformed) data; Values in parenthesis are transformed by arcsine transformation.

versicolor were the most susceptible to infection by *B. bassiana* Strain 687, but were resistant to infection by *B. bassiana* Strain 513reTgr. Similar to the current results, effectiveness of *B. bassiana isolates* against *T. versicolor* were also reported by Draganova et al. (2012) who found that the isolate *B. bassiana* 687Bb caused cumulative percentage mortality confirmed by mycosis reaching 98.8 and 100% by day 7 and 10, respectively. The efficacy of *P. lilacinum* against other insects was reported by Angelo et al. (2012) and Medeiros et al. (2018) which caused 100% mortality in *Aleurocanthus woglumi* nymphs. Similarly, Barson (1977) reported that *B. bassiana* caused 100% mortality of elm bark beetle, *Scolytus scolytus* (L). The slight variation from the previous pathogenicity results can be explained by several factors, including the strain of the fungus, ability of the host's defense mechanism to overcome the infection, quantity of spores, and the environmental conditions. The current results showed that most of the local isolates can produce high mortality among 3rd instar larvae of *T. granarium* 7 days after inoculation under lab conditions. Infected 3rd instars larvae of *T. granarium* were very inactive after treatment and move very slowly unless touched with their body covered with fungal mycelium. This temporary inactivity of the larvae was also noted by Malik (1991) in *Maruca testulalis.* On the other hand, coverage of the treated larvae by the white mycelia was also reported by Barson (1977) who showed that aerial mycelium first appeared on the dead larvae of *S. scolytus* (L.) infected with *B. bassiana* and gradually covered the body surface. The Probit analysis of data obtained from the current experiments showed that the increase in the exposure period increases the efficacy (decrease LT_{50} and LT_{90} values) and decreases their ratios as well as the slope of mortality regression lines which became steeper indicating improvement in the homogeneity of responses.

The current results reported that most of the test isolates induced slightly higher LT_{50} (4.17-6.68 days) values compared to those reported by Draganova et al. (2012) who reported that the isolate 689Bb was the most virulent to the larvae of *T. versicolor* with LT_{50} values of 3.4 days.

The genomic classification done in the current study indicated a polymorphisms among the test nine isolates in the ITS region shown in amplified fragment of 570 bp (base pair). Some sequence variation was detected within the ITS region, previously reported by Wada et al. (2003) and Rehner and Buckley (2005).

Conclusions

Nine isolates effective isolates of local entomopathogenic fungi were isolated from soil collected from different sites in Sudan by the enrichment culture techniques. Most of the isolates were found effective against the 3rd instars larvae of Khapra beetle and three of them gave results comparable to the standard treatment. The LT_{50} values of *B. bassiana* isolate Sud afro.18 (MK046654) and *M. anisopliae* isolate Dmazeen F1 (MK046658) are comparable to that induced by the commercial product

Table 2. Log-time probit response of the 3rd larval instars of *Trogoderma granarium* dipped in the suspension of the local isolates of entomopathogenic fungi and commercial product Eco Bb®.

Isolate	Lethal time (days)		Fudicial limits		LT_{90}/ LT_{50} ratio	χ^2	Slope	D.F	Relative potency
			Lower (95%)	Upper (99%)					
Eco-Bb® product	LT_{50}	4.17	3.78	4.59	2.38	28.00	0.294	5	0.62
	LT_{90}	9.93	8.35	12.74					
M. anisopliae MK046658	LT_{50}	4.23	3.92	4.58	1.94	21.70	0.231	5	0.63
	LT_{90}	8.23	7.36	9.99					
B. bassiana MK046652	LT_{50}	4.27	3.95	4.61	1.96	29.31	0.228	5	0.64
	LT_{90}	8.37	7.37	9.98					
B. bassiana MN598664	LT_{50}	4.85	4.47	5.31	2.09	10.91	0.250	5	0.73
	LT_{90}	10.15	8.65	12.83					
B. bassiana MK046654	LT_{50}	4.96	4.60	5.40	1.97	16.57	0.229	5	0.74
	LT_{90}	9.78	8.43	12.11					
P. lilacinum MK046655	LT_{50}	5.15	4.70	5.73	2.27	7.73	0.277	5	0.77
	LT_{90}	11.69	9.66	15.51					
A. viridis MK046656	LT_{50}	5.20	4.67	5.91	2.64	27.18	0.329	5	0.78
	LT_{90}	13.72	10.85	19.60					
B. bassiana MN598665	LT_{50}	5.93	5.38	6.73	2.21	6.75	0.269	5	0.89
	LT_{90}	13.12	10.58	18.43					
B. bassiana MN598666	LT_{50}	6.60	5.86	7.81	2.41	16.22	0.298	5	0.99
	LT_{90}	15.93	12.17	24.91					
M. anisopliae MK046659	LT_{50}	6.68	5.78	8.25	3.12	12.77	0.385	5	1.00
	LT_{90}	20.86	14.79	36.93					

χ^2 ; Chi-square, DF; degree of freedom, LT_{50}; Lethal time that induce 50% mortality in a test population, LT_{90}; Lethal time that induce 90% mortality in a test population.

Eco-Bb®. The mycosis tests confirm that incubation by the isolates is the causal agent of mortality of the dead larvae. The study recommends further work in refining the dosage range, evaluation of applicable test method and validation of the results under real storage conditions. Future work can also include; the study of any possible side effects of the isolates on consumers of treated products; consumer perception to treated products and formulation of the products in simple applicable formulation to be used in small storage facilities in Sudan and other developing countries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Metabolic engineering of *Neurospora crassa* for increasing carotenoids synthesis

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Carotenoids are essential nutrient compounds with numerous biological functions. *Neurospora crassa* is a model filamentous fungus with orange pigmentation which is attributed to the accumulation of carotenoids containing neurosporaxanthin (NX) and neutral carotenoids (NC). To enhance carotenoids synthesis in *N. crassa*, isoprene diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) were increased using the genes, xylulose-5-phosphate phosphoketolase (*XPK*), phosphotransacetylase (*PTA*), and NADH-specific-3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGR*), as single, fused and three combined expressions to channel more carbon source into the mevalonate pathway (MVP). The single (*PTA*, *XPK*, *HMGR*), fused (*PTA:HMGR* & *XPK:HMGR*) and three combined gene (*PTA* with fused *XPK:HMGR*) expressions in engineered fungal resulted in carotenoid titers with contents of NX accumulated up to 4.5 mg/g DW and NC up to 1.7 mg/g DW as compared to the wild-type with NX up to 1.54 mg/g DW and NC up to 0.8 mg/g DW. The optimized MVP with metabolic engineering methods is a key method to increase the synthesis of carotenoid and other active terpenoids in *N. crassa*.

Key words: *Neurospora crassa,* mevalonate pathway, carotenoids, neurosporaxanthin, neutral carotenoids.

INTRODUCTION

Carotenoids, which are a general term for xanthophylls and carotenes depending on weather they contain or lack oxygen in their molecules, can be found in fungi, plants and algae (Lee et al., 2012). They have numerous biological functions in the human body. In the food industry, carotenoids are used in many products such as cheese, pastry and some non-alcoholic beverages. It is also used as an active ingredient in cosmetic products as it provides protection against UV radiation exposure (Barreiro and Barredo, 2018). Originally, carotenoid was extracted from plants, over the years, various classifications of carotenoids have been synthesized through microbial sources such as bacteria (Yoon et al., 2007, 2009), yeast (Marova et al., 2012; Simova et al., 2004) and algae (García-González et al., 2005). The rising market demand of the various classifications of carotenoids has stimulated the development of its diverse production methods. Although current titers in many engineered fungi hosts except *Fusarium fujikuroi* (Parra-Rivero et al., 2020) still lack economic feasibility, future

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research leading to better approach in alternative hosts may be established for large scale production with less cost.

Neurospora crassa is a filamentous orange bread mold whose potential as a model organism has been recognised for several reasons (Davis and Perkins, 2002). It is known to have a relatively simple life cycle, rapid growth rate and ease of propagation on cheap growth media. Its haploid nature allows for dominant and recessive traits to be expressed in their offsprings (Turrà et al., 2016). It is non-toxic and is traditionally used for food production in south eastern Asia and is considered safe for other technical applications (Perkins and Davis, 2000) making it a suitable strain for synthetic biology. It also has an added advantage where it can grow on discarded lignocellular biomass for itself secreting cellulose and semi-cellulose degrading enzymes (Collier et al., 2020; Liu et al., 2020), and is more tolerant (Feldman et al., 2019; Tiwari et al., 2018) in active synthesized terpenoidal compounds such as steroidal or triterpenoid saponins which has a potential of reducing future production cost of active terpenoid compounds used in pharmaceutical or functional food industry. Neurosporaxanthin (NX) a carboxylic xanthophyll is the major carotenoid product of *N. crassa* which is usually accumulated with varying amounts of its neutral carotenoids (NC) (Hornero-Méndez et al., 2018). NX provides the typical orange pigmentation to *N. crassa* because of its accumulation in the airborne spores (Avalos et al., 2012).

In the mevalonate pathway (MVP) for terpenoid synthesis, isoprene diphosphate (IPP) and its interconvertible isomer, dimethylallyl diphosphate (DMAPP), are key intermediate metabolites which are accumulated starting with acetyl-CoA in non-photosynthetic hosts, hence, our optimized synthetic strategy in an engineered fungus by accumulation of acetyl-CoA and channelling its carbon flux into MVP (Moser and Pichler, 2019). In this study, 3 genes; Xylulose-5-phosphate phosphoketolase (*XPK,*NCBI: CP000414.1:1934181-1936622), phosphotransacetylase (*PTA*, ABAJ01000001.1:3408314-3409315) and NADH specific-3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGR*, CP000032.1:179002-180303) as single genes, fused (*PTA:HMGR* and *XPK:HMGR*) and three combined gene expressions (*PTA* with fused *XPK:HMGR*) were introduced into *N. crassa* with the aim of channelling more carbon source to acetyl-CoA by concert action of XPK and PTA, then acetyl-CoA conversion to IPP and DMAPP in MVP was enhanced by NADH-specific HMGR.

MATERIALS AND METHODS

Strains, plasmids and chemicals

The 301-6 strain, mating type “A” with a mutated his-3 gene (Δhis, cannot grow without histidine) is the *N. crassa* strain used in this study. Other strains and plasmids used in this study are listed in Table 1. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, (Woburn**,** USA). Common reagents and kits for molecular cloning were purchased from Shangon Biotech (Shanghai, China).

Cultural media and growth conditions

Cloning host, *Escherichia coli* (DH5α)*,* was grown on LB media with Ampicillin antibiotics. The 301-6 strain was cultivated on minimal slants supplemented with histidine (20 ml/L 50x Vogel’s (Vogel, 1956), agar 15 g/L, sucrose 30 g/L and histidine 0.15 g/L) for four days at 30°C under the condition of light and the spores were harvested with distilled water. To improve carotenoid synthesis, fermentation was carried out with 3 different methods. Method 1: fermented with media1 (20 ml/L 50x Vogel’s, 20 g/L glucose), method 2: fermented with media 2 (50 ml/L 50x Vogel’s, 50 g/L glucose), and method 3: after 4 days inoculation in media 2, additional 2% glucose was added at 30°C at 160 rounds per minute (rpm) in the shaker incubator.

Construction of expression vectors

The pPcfp_Myc_His and pPcfp_Myc_HyR with constitute expression promoter of cfp (Alvarez et al., 1993) pyruvate decarboxylase (NCBI: NC_026507.1:3384904-3387654), with *his-3* and hygromycin selection markers, respectively were used as starting vectors for construction (Table 1). After, the codon was optimized for expression in *N. crassa*, the coding sequence of *XPK* (2484 bp) from *Leuconostoc mesenteroides* subsp*. mesenteroides* ATCC 829, *PTA* (1049 bp) from *Clostridium kluyveri* DSM 555 and *HMGR* (1349 bp) from *Ruegeria pomeroyi* DSS-3 with their start codon (ATG) up flanking with a fused sequence of “GGCGGTGCTGCA” coding for peptide linker “Gly_Gly_Ala_Ala” were obtained by chemical synthesis from Shangon (Shanghai, China). To construct the single gene expression vectors, *XPK*, *PTA* and *HMGR* were amplified by PCR with primer pairs of XPK_P1 & XPK_P2, PTA_P1 & PTA_P2 and HMGR_P1 & HMGR_P2, respectively to add *BamH* I site on 5′ end and both *Bgl* II and *EcoR* I sites on 3′ end (Table 2). After being digested with both *BamH* I and *EcoR* I, the fragments were inserted into *Bgl* II and *EcoR* I sites of pPcfp_Myc_His to get intermediate vectors, pPcfp_XPK_His, pPcfp_PTA_His, and pPcfp_HMGR_His (Table 1). Meanwhile, the terminator, glyceraldehyde-3-phosphate dehydrogenase (NCBI: NC_026502.1:3180104-3182245) of *N*. crassa (Shinohara et al., 1998) was amplified with primer pairs of T_gdph_P1 & T_gdph_P2, and the amplified *T_gdph* fragment was further digested with restriction enzymes *Bgl* II and *EcoR* I, followed by insertion into the intermediate vectors (pPcfp_XPK_His, pPcfp_PTA_His, and pPcfp_HMGR_His) to get the final expression vectors pXPK, pPTA and pHMGR, respectively (Table 1, Figure 2A).

For the construction of two fused gene expression vectors, the *HMGR* was re-amplified with primer pairs HMGR_P1 & HMGR_P2 (Table 2), digested with both *BamH* I and *EcoR* I and inserted into *Bgl* II and *EcoR* I sites of pPcfp_XPK_His and pPcfp_PTA_His to get fused expressed intermediate vectors, pPcfp_XPK:HMGR_His and pPcfp_PTA:HMGR_His. The *T_gpdh* of *N. crassa* was inserted into the fused expressed intermediate vectors (pPcfp_XPK: HMGR_His and pPcfp_PTA:HMGR_His) after it was digested with both *Bgl* II and *EcoR* I to get the final fused vector pXPK:HMGR and pPTA:HMGR (Table 1, Figure 2A). The pPTA_H vector with hygromycin resistant marker was constructed for combining aforementioned three gene expressions in *N. crassa.* It was constructed by inserting the CDS of *PTA*, followed by *T_gdph* fragment into pPcfp_Myc_HyR (Table 1 and Figure 2A).

Table 1. Strains and plasmids used in this study.

Name of plasmids/strains	Characteristics	Source
pPcfp_Myc_His	*cfp* promoter, Histidine resistance (His)	He Qun China Agricultural University
pPcfp_Myc_HyR	*cfp* promoter, hygromycin resistance (HyR)	lab's collection
pPcfp_XPK_His	With Linker (GGCGGTGCTGCA encoding Gly_Gly_Ala_Ala)	This study
pPcfp_PTA_His	same as below	This study
pPcfp_HMGR_His	same as below	This study
pPcfp_PTA:HMGR_His	same as below	This study
pPcfp_XPK:HMGR_His	same as below	This study
pPcfp_PTA_HyR	same as below	This study
pXPK	same as below	This study
pPTA	same as below	This study
pHMGR	same as below	This study
pPTA:HMGR	same as below	This study
pXPK:HMGR	same as below	This study
pPTA_H	same as below	This study
*Escherichia coli (*DH5α)	Cloning host (Δ lac)	Lab's collection
Neurospora crassa (301-6, A)	Fugal host (Δ his-3)	Lab's collection
PTA/NC_{301-6}	pPTA transformed N. *crassa* 301-6 Strain	This study
XPK/NC_{301-6}	pXPK transformed N. *crassa* 301-6	This study
HMGR/NC_{301-6}	pHMGR transformed N. *crassa* 301-6	This study
PTA_HMGR/NC_{301-6}	pPTA_HMGR transformed 301-6 N. *crassa* 301-6	This study
XPK_HMGR/NC_{301-6}	pXPK_HMGR transformed 301-6 N. *crassa* 301-6	This study
PTA & XPK_HMGR/NC_{301-6}	pPTA_H and pXPK:HMGR co-transformed N. crassa 301-6	This study

Transformation of *N. crassa*

The single or fused gene expression vectors were transformed into the 301-6 strain using electroporation as described by Vela-Corcía et al. (2015) with some modifications. The electroporated *N. crassa* spores were then inoculated on minimal agar media and incubated at 30°C. The histidine free growing fungal colonies were picked unto minimal slants to grow again without histidine at 30°C for about 4 to 6 days. Their respective genomic DNAs were extracted from new growth colonies as described by Zhang et al. (2010) were used as template, and the transformed strains were finally confirmed by amplifying the inserted genes with detecting primers XPK_P1 & XPK_P2, PTA_P1 & PTA_P2 and HMGR_P1 & HMGR_P2 (Table 2, Figure 2B). For combing three genes in *N. crassa*, the expression vector of pPTA_H was transformed again into the successfully confirmed *XPK:HMGR* strain by selection on minimal agar media with hygromycin, and the strains were further confirmed by PCR amplification.

Total RNA of the transformed and wild-type strains were extracted using the Hi pure RNA isolation kit from Magen (Beijing, China) according to the manufacturer's instructions. The qualified isolated RNA was then transcribed into complimentary DNA (cDNA) using Fastking gDNA dispelling RT supermix from Tiangen (Beijing, China) according to the manufacturer's protocol. The primers XPK_D_P1&XPK_D_P2, PTA_D_P1&PTA_D_P2, HMGR_D_P1& HMGR_D_P2, PTA_H_D_P1& PTA_H_D_P2, and XPK_H_D_P1& XPK_H_D_P2 (Table 2) were used to amplify the fragment, respectively to detect gene expressions with the cDNA derived from mRNA as templates respectively (Figure 2C).

Growth rate determination

Fermentation for growth rate determination was carried out in a 500 mL erlenmeyer flask with 100 mL media and 1 mL of spore suspensions (10^6 spores/mL) of transformed and wild type strains obtained from minimum slant media

growth fungi was inoculated into the 3 different fermentation media, respectively (see cultural media and growth conditions) at 30°C with rotation of 160 rpm having 3 replications. During the cultivation, replicate flasks were drawn from the shaker cultivated at 5 different sampling time (48, 96, 144, 192 and 240 h), and their mycelia were filtered and dry weights determined after drying at 80°C to constant weight. The growth curves of the strains were then recorded from their dry weights (g/L) versus incubation time in hours.

Carotenoid quantification using different concentrations of carbon source

Fermentation was further carried out with only media 1, media 2 and starting from media 2 then adding glucose to final concentration of 2% (see culture media and growth conditions) after 4 days inoculation to investigate carotenoids titers among engineered and natural strains. Carotenoids were extracted from the accumulated biomass by bleaching with methanol after they have been weighed and ground with sea sand and the NX and NC concentrations were determined using the subtraction protocol as described by Avalos et al. (2012) with at least three independent duplications. NX concentration was estimated using its widely accepted specific Extinction Coefficient 1715 (Avalos et al., 2012) while NC concentration was estimated using β-carotene purchased from Jinang technology (Kunming, China), as standard with the measured specific Extinction Coefficient of 2590.

Microscopic observations of spores

Spore samples were picked directly from the solid media of different strains with nearly the same concentration by suspension in 80% glycerol, and a tiny drop of the suspended spores of about (10 µL) were viewed under the microscope.

Statistical analysis

Data was analysed using GraphPad prism version 9.0 (California, USA). The data was reported as means of each strain and statistical significance determined using analysis of variance (ANOVA). The level of statistical significance was set at ($p < 0.05$) with Tukey's multiple comparism analysis for strains that showed significance.

RESULTS

Verification of gene expression in *N. crassa*

The engineered strains were constructed to increase either only acetyl-CoA with single gene expression (*XPK* or *PTA*), increase only MVP influx by *HMGR* or both acetyl-CoA and MVP (Figure 1) with 2 fused gene expression (*XPK:HMGR, PTA:HMGR)* or 3 gene co-expression *(PTA with XPK:HMGR)* (Figure 1).

The constructed expression vectors (Figure 2A) were transformed with the method of electroporation and selected on minimal media. The histidine/hygromycin selected fungal colonies were further confirmed as transformed strains by PCR (Figure 2B) using primer pairs from Table 2. Expressions of single gene *PTA*, *XPK & HMGR*, fused *PTA:HMGR & XPK:HMGR* gene and *PTA* with the fused *XPK:HMGR* in *N. crassa* were confirmed (Figure 2C) at the mRNA level with primers using RT_PCR amplification (Table 2).

Changes in growth rate

The engineered *N. crassa* strains, either as single expressions of *XPK, PTA* & *HMGR*, fusion expressions of *PTA:HMGR* & *XPK:HMGR* or co-expression of *PTA* with *XPK:HMGR* were grown with media 1, media 2 and first grown in media 2 than adding final 2% glucose 4 days after inoculation, respectively. The engineered *N. crassa* strains did not reduce the growth rate (Figure 3A, 4A and 4B), as compared with 301-6 strain transformed with an empty vector, pPcfp_Myc_His (Table 1), and nearly all the transformed strains cumulated higher biomass with the strain of co-expression genes of *PTA* with fused *XPK:HMGR* having the highest biomass accumulation (7.86 g/L). When the strains were grown in media1 the exponential growth started after 48 h inoculation and entered into stationary phase after 96 h cultivation. When the strains were grown in media 2 (Figure 3A), the biomass accumulation increased about 2 times as compared with media 1 cultivation, however, the same single "S" pattern of growth was observed as cultivated in media 1. The growth pattern was changed to nearly double "S "type with the highest biomass accumulated recorded 192 h after cultivation first with media 2 and additional final 2% glucose media 4 days after inoculation. This indicated that, the biomass accumulation of the strain is dependent on available carbon source. The increase in the biomass of the engineered strains may be due to the channelling of the intermediate metabolites to terpenoid synthesis partially reducing the primary metabolic feedback inhibition and/or accumulation of terpenoids (such as carotenoid) increasing the stress tolerance of strain in fermentation condition.

NX and NC quantification in engineered strains

Using media 1 as fermentation broth, a preliminary test was carried out to quantify NX and NC; 96 h after inoculation just as the end of exponential growth of the mycelial dry weight was recorded and 144 h when the highest biomass accumulation was recorded (Figures 3A, 4A, and 4B). The 144 h after cultivation was used as the harvesting time from the preliminary testing as it had a significant increase in carotenoid concentrations as compared to that after 96 h (Figure 3B). NX concentrations had titers up to 0.81, 0.76, 1.0, 1.08, 1.11 and 1.39 mg/g with the wild-type having 0.67 mg/g for 96 h while 144 h had NX titers up to 1.09, 1.09, 1.3, 1.55, 1.66 and 1.83 mg/g with the wild-type having 0.78 mg/g. NC concentrations had titers up to 0.28, 0.25, 0.38, 0.47, 0.54 and 0.75 mg/g with the wild-type having 0.14 mg/g

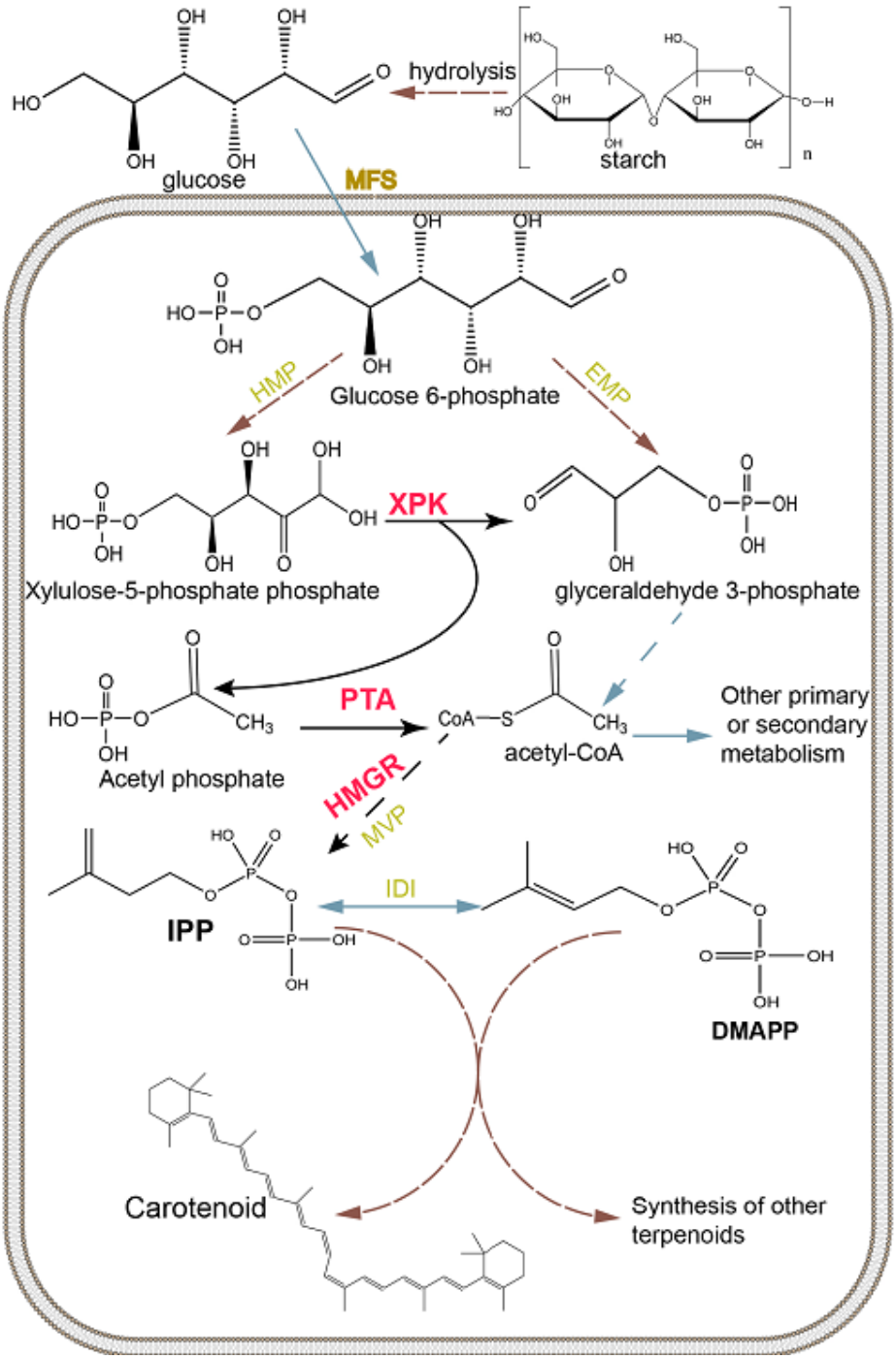

Figure 1. The metabolic engineered *N. crassa* pathway for acetyl-CoA accumulation with expression of *XPK* and *PTA* and increasing IPP and DMAPP by *HMGR* expression for enhancing carotenoid biosynthesis. MFS: Major facilitator superfamily for out cellular sugar transporting, HMP: hexose monophosphate pathway, EMP: Embden-Meyerhof-Parnas pathway, MVP: Mevalonate pathway, IDI; isopentenyl-diphosphate Delta-isomerase, *XPK, PTA* and *HMGR* expression highlighted by red.

for 96 h while 144 h had NC titers up to 0.56, 0.56, 0.65, 0.75, 0.81 and 0.93 mg/g with the wild-type having 0.33 mg/g cultivated with media 1, respectively (Figure 3B and 3C). Fermentation carried out with only media 2 had NX titers up to 1.25, 1.27, 1.72, 2.1, 2.3 and 2.66 mg/g with the wild-type having 1.0 mg/g while NC concentrations

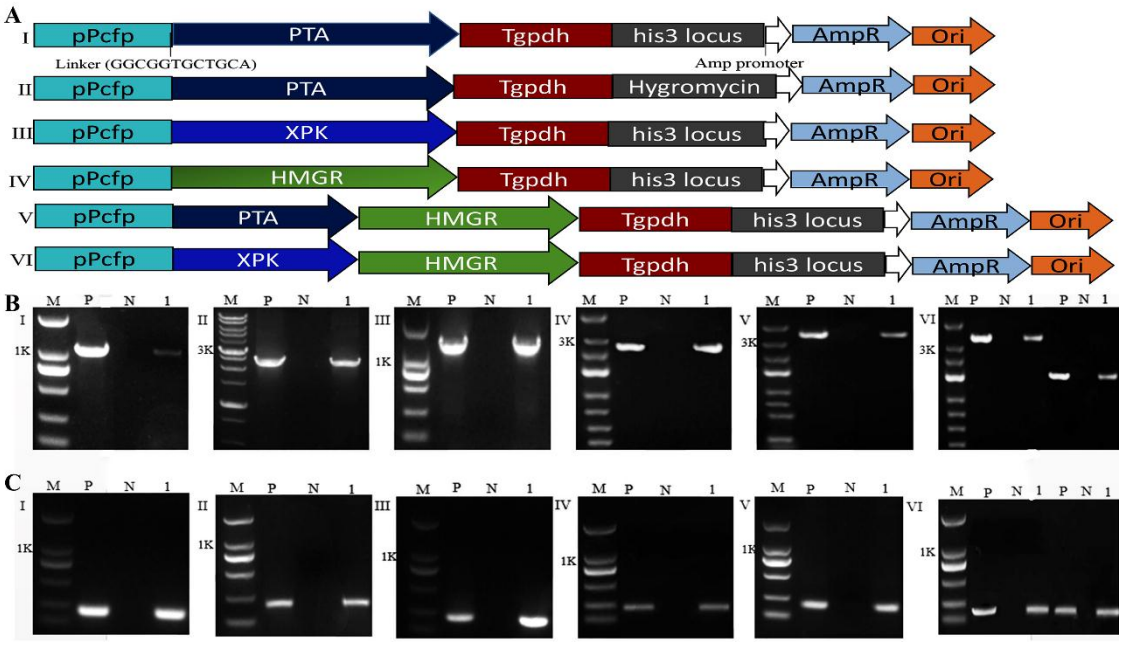

Figure 2. Construction of engineered strains of *Neurospora crassa*. (A) Linear representation of chimeric genes as constructed expressions driven by cfp promoter, a designed sequence linker of "*GGCGGTGCTGCA*" coding peptide of "Gly_Gly_Ala_Ala" for fusion protein link as shown on the chimeric genes; (A I) *PTA*, (A II) *PTA* (with hygromycin resistance), (A III) *XPK*, (A IV) *HMGR*, (A V) fused *PTA:HMGR* and (A VI) fused *XPK:HMGR* with a gpdh terminator of *N. crassa.* (B) Agarose gel electrophoresis of amplified genes from transformed strains. Lane M: 2k, 5k &10k bp DNA ladder, lane P: positive control, lane N: negative control and lane 1: amplified genes from the genomic DNA of the transformed strains where (B I) *PTA* (1049 bp), (B II) *XPK* (2484 bp), (B III) *HMGR* (1349 bp), (B IV) fused *PTA: HMGR* (2393 bp), (B V) fused *XPK: HMGR* (3533 bp) and (B VI) PTA with fused XPK: HMGR (1049 and 3533 bp). (C) Agarose gel electrophoresis of RT_PCR amplified genes from engineered strains. Lane M: 2k bp DNA ladder, lane P: positive control, lane N: negative control and lane 1: amplified genes from the cDNA derived from mRNA of the transformed strains where (C I) *PTA* (196 bp), (C II) *XPK* (198 bp), (C III) *HMGR* (188 bp), (C IV) fused PTA: HMGR (*194*bp), (C V) fused *XPK: HMGR* (168bp) and (C VI) *PTA* with fused *XPK:HMGR* (196 and168 bp), respectively.

had titers up to 0.66, 0.66, 0.75, 0.85, 0.91 and 1.07 mg/g with the wild-type having 0.44 mg/g. (Figure 4C). NX and NC concentrations increased significantly starting from media 2 then adding additional 2% glucose 4 days after inoculation as compared to using only media 2 or media 1, this strategy resulted in NX titers up to 2.54, 2.58, 2.75, 2.97, 3.1 and 4.5 mg/g of dry weight, respectively as compared to the wild-type with 1.70 mg/g after 192 h and NC titers up to 1.06, 1.05, 1.19, 1.27, 1.30 and 1.54 mg/g of dry weight, respectively as compared to the wild-type with 0.80 mg/g after 192 h (the highest biomass accumulation) (Figure 4D).

Phenotypical changes of engineered strains of *N. crassa*

Although there was no changes in mycelial growth among the natural and transformed strains, other phenotypical changes were observed such as deep colouration among transgenes, which is consistent with the increase in carotenoid concentrations determined by its colour enrichment, morphological changes in spores observed under the microscope showed that, the non-transformed strain had jointed spores with regular cell shapes, while that of the transformed strains showed dis-jointed spores with irregular cell shapes. This may be attributed to the insertion of the gene expressions to channel more carbon metabolic flux for terpene synthetic pathway, resulting in reduced polysaccharides biosynthesis of the cell wall leading to weak connections between cells. The irregular shapes of the cells of the transgenic strains may be attributed by its individualistic nature of weak cell wall as compared to the attached with branched appearance of the non-transgenic strain (Figure 5), and the weak wall property may have promoted germination of spores speeding biomass accumulation.

DISCUSSION

With an established easy genetic transforming system, the filamentous fungus *N. crassa*, which has a high

Table 2. Primers used in this study.

Primer name	Primer sequence (5' -3')	ize (bp)
PTA_P1	CGC***GGATCC***ATGGATCC**GGCGGTGCTGCA**ATGG (bold and italic shows *BamH* I site and bold underlined shows linker for encoding Gly_Gly_Ala_Ala, same as below)	1049
PTA_P2	CGC**GAATTC**TCATTA**AGATCT**GCCCTGAGCC (bold and italic show shows *EcoR* I &*Bgl* II sites respectively, same as below)	
XPK_P1	GCA***GGATCC***ATGTCC**GGCGGTGCTGCA**ATGG	2484
XPK_P2	CCG***GAATTC***TCATTA***AGATCT***CTTGAGGGAC	
HMGR_P1	CGC***GGATCC***ATGGATCC**GGCGGTGCTGCA**ATGG	1349
HMGR_P2	GCA***GAATTC***TCATT*A****AGATCT***GGTGTTCTC	
T_GPDH_P1	CGC***AGATCT***ATGTCGGTTGCGTACCCGCG	719
T_GPDH_P2	GC***GAATTC***AGCGGGCGGCAAGCGGAT	
PTA_D_P1	GCTCCGAGTCCGTCATCAAG	196
PTA_D_P2	ATCATGGTGGCGAAGTAGATGG	
XPK_D_P1	GCACTCCATCAAGTTCCTCAAC	198
XPK_D_P2	TGGTGGTAGGTGGCGTAGT	
HMGR_D_P1	GATATCGAGGTCCACGTCTTCC	188
HMGR_D_P2	AGATCGGCGAGGTTGGAGAG	
PTA_H_D_P1	ACTCCCGCATCGAGAAGAT	194
PTA_H_D_P2	GAACTTGCCGATGACGTTC	
XPK_H_D_P1	TCAAGATGACCGGCAAGAC	168
XPK_H_D_P2	GATGACGTTCTCGATCATGC	

proliferation rate, relatively simple life cycle and is able to grow with cheap media has played significant roles in numerous biotechnological applications including production of important secondary metabolites (Moser and Pichler, 2019). Since it can produce carotenoids naturally, the aim of this study is to optimized IPP and its interconvertible isomer DMAPP accumulation for efficient interested terpenoids biosynthesis from relative simple carbon source such as glucose with easy detection of carotenoid colour enrichment. The *XPK*, catalyses the formation of acetyl phosphate and glyceraldehyde-3-phosphate from xylulose-5-phosphate (Meadows et al., 2016), the PTA, catalyses the reversible reaction between acetyl phosphate and Acetyl-CoA (Castaño-Cerezo et al., 2009), while HMGR, enhancing the conversion of Acetyl-CoA to IPP and DMAPP by MVP (Istvan, 2002), were expressed as single, fused or triple genes together in *N. crassa* to convert more media carbon source to Acetyl-CoA followed by forming IPP and DMAPP (Figure 1). The engineered *XPK* and/or *PTA* overexpression strains not only

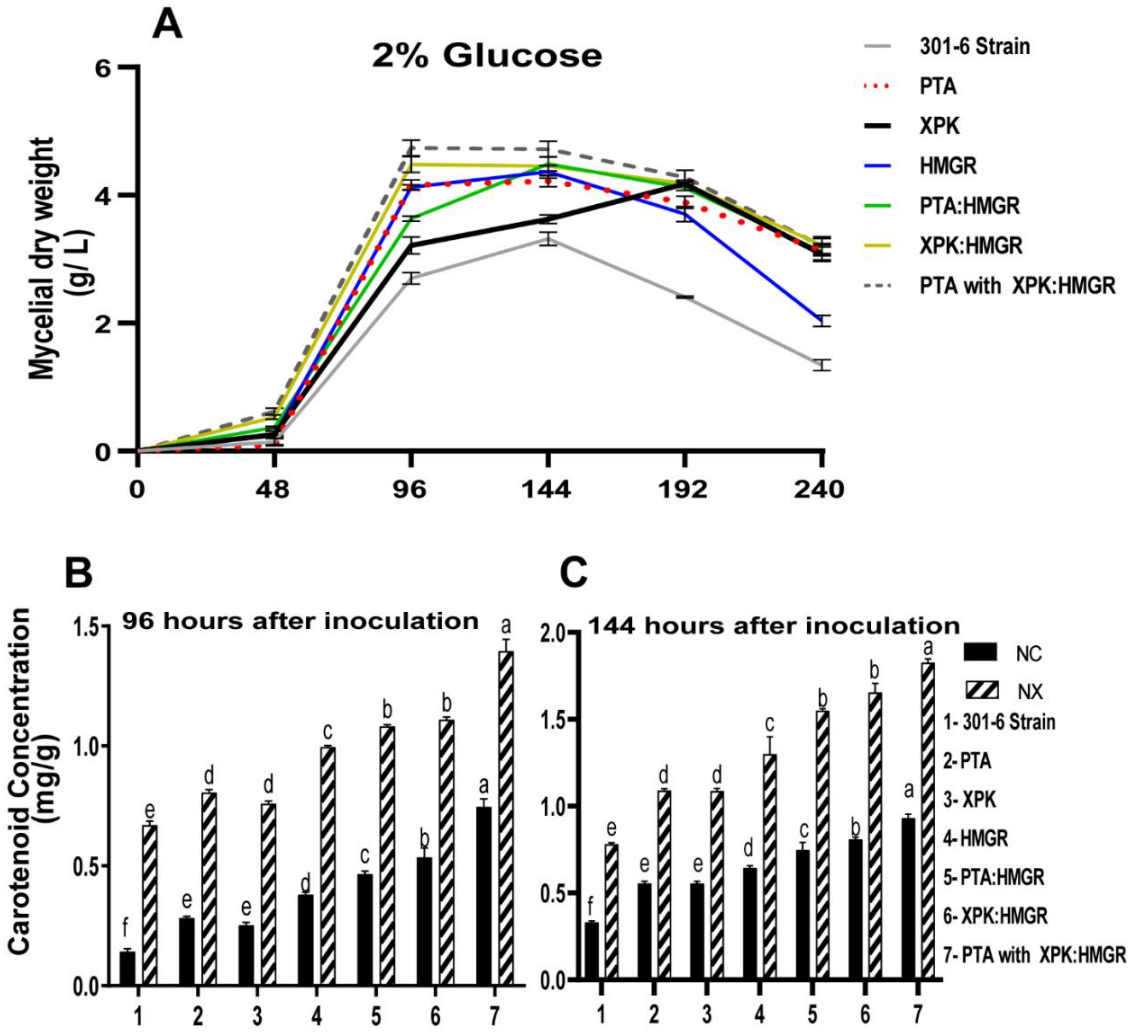

Figure 3. Changes in growth rate among metabolic engineered and wild type strains of *N. crassa*. (A) Growth rate of the transgenes and wild-type using media 1 (2% glucose) from spores at 5 different sampling times (48, 96, 144,192 and 240 h) with at least three independent replications. Carotenoid concentration (NX and NC) titers determined among the engineered and wildtype at (B) 96 h after inoculation just as the first exponential growth was recorded, (C) 144 h after inoculation when the highest biomass accumulation was recorded using media 1 respectively. Bars which do not share the same letters are significantly different with p-values <0.05.

produces more Acetyl-CoA, but also reduce acetic acid accumulation and relieve feedback inhibition of glycolysis, while the HMGR normally use the co-enzyme NADPH and not NADH as its co-enzyme (Beach and Rodwell, 1989; Istvan and Deisenhofer, 2001), however, the HMGR used in this study has the NADH-specific co-enzyme thus (Meadows, 2016), its may channel more carbon flux and energy into MVP responsible for carotenoid biosynthesis for NADH is more abundant than NADPH, but also speeding the recycling between NADH and NAD^+ to remove the glycolysis feedback inhibition by NADH in hyperopic condition. Additionally, the synthesis of carotenoids may have increased the stress tolerance during the growth; these changes in the engineered strains are consistent with the results that all engineered *N. crassa* strains did not reduce the growth rate by transgenes burden, as engineered strains increased their biomass as compared to the non-transgenic wild type (Figures 3 and 4).

Aside the no change in mycelial growth among the strains, dis-jointed spores with irregular cell shapes were observed and may be attributed to the inserted transgenes channelling the more carbon metabolic flux of the β-carotenoids synthesis pathway, resulting in reduced polysaccharides biosynthesis of their cell walls leading to weak connections between cells (Figure 5). As we know, this is the first time, to significantly increase carotenoids by optimizing MVP by channelling the carbon source to synthesize IPP and DMAPP in *N. crassa*. Our engineered N. crassa increased 2~3 folds of carotenoids compared

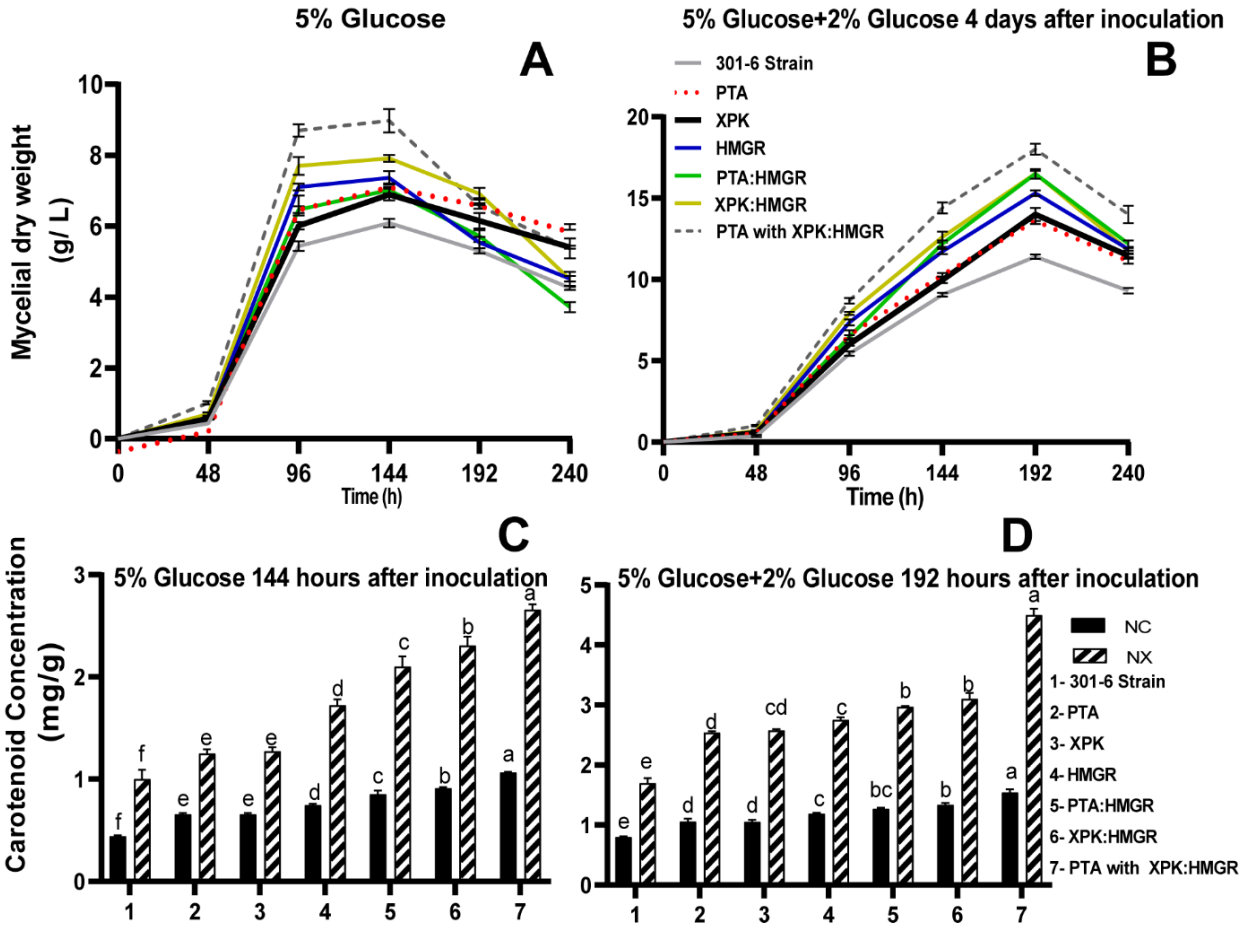

Figure 4. Changes of carotenoid concentration among engineered and wild type strains of *Neurospora crassa*. (A) Growth rate of the transgenes and wild-type using media 2 (5% glucose) and (B) starting from media 2 then add additional 2% glucose 4 days after inoculation from spores at 5 different sampling times (48, 96, 144,192 and 240 h) with at least three independent replications. Carotenoid concentration (NX and NC) titers determined among the engineered and wild type strains at (C) 144 h after inoculation using only media 2 and (D) 192 hours after inoculation starting from media 2 then add additional 2% glucose 4 days after inoculation from transgenes and wild-type strains. Bars which do not share the same letters are significantly different with p-values <0.05.

with wild type, with titers up to 4.5 mg/g for NX and 1.54 mg/g for NC. The currently achieved content of carotenoids in these strains do not reach the popular production in engineered *Fusarium fujikuroi* which produced the highest NX titers up to 8.3 mg/g dry weight (Parra-Rivero et al., 2020), however, the engineered *N. crassa* stains are not fully adapted, and their fermentation broth are not optimized for increasing biomass accumulation for carotenoids production, thus, continuing with a rational design coupled with adaptation and batch fermentation at the early stages, adding more nitrogen source for cell mass accumulation, adding more carbon source at stationary phase and improving the aerobic condition during fermentation may finally catch up to the carotenoids yield achieved in higher yield filamentous fungi or even catch the yields of yeast and the *E. coli*. It is very important as our final goal of the engineered *N. crassa* is to make this fungus a working horse to synthesize other active terpenoids, and this can be achieved by targeting terpenoids synthetic pathway and switching off carotenoid synthesis after the MVP pathway is optimized by carotenoid colour indication.

Conclusion

Optimized mevalonate pathway (MVP) by metabolic engineering is a key step to synthesize carotenoid and other active terpenoids in *N. crassa* as our engineered *N. crassa* strains increased 2~3 folds of carotenoids compared with wild type.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

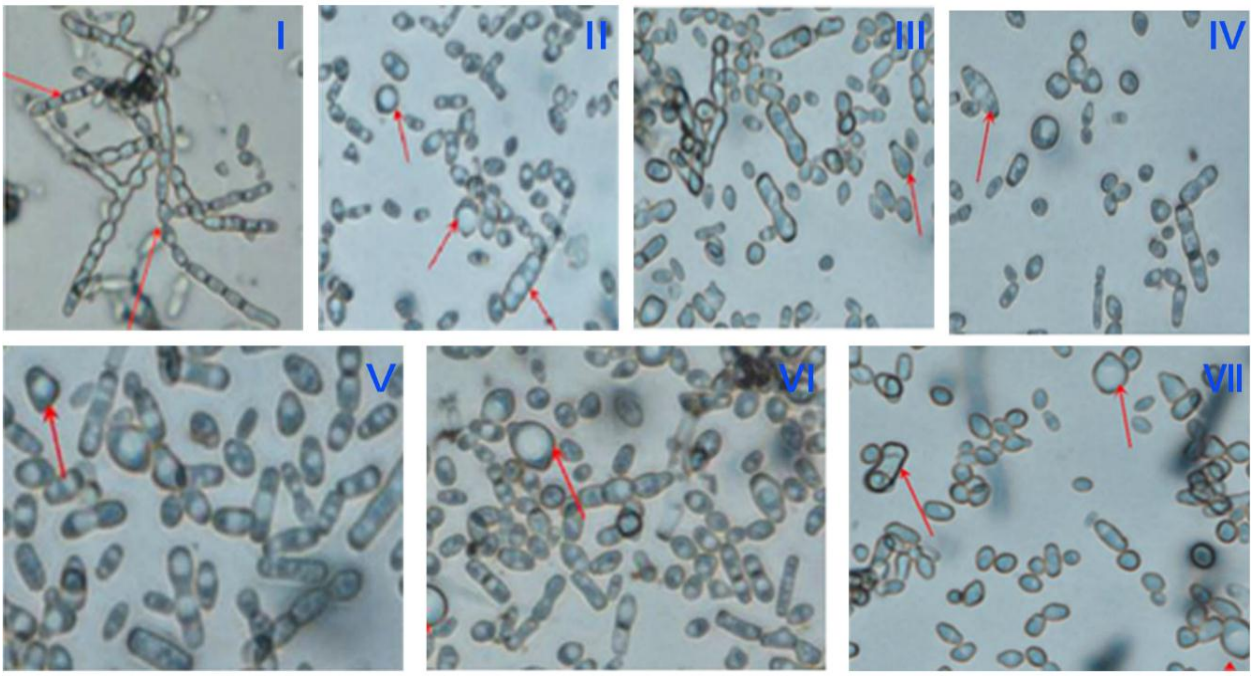

Figure 5. Changes spore phenotype among engineered and wild type strain. (I) 301-6 strain spores viewed under the microscope showing jointed cells shapes and red arrows showing regular cell shapes and sizes. (II~VII) transformed strains (*PTA, XPK, HMGR*, fused *PTA:HMGR*, fused *XPK:HMGR* and *PTA* with fused *XPK:HMGR* strains, respectively) spores viewed under the microscope showing different dis-jointed cells with the red arrows showing irregular cell shapes and sizes.

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Optimization of PCR protocol for ISSR marker based genetic diversity assessment of acid lime [*Citrus aurantifolia* (Christm.) Swingle] germplasm in Eastern Nepal

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Acid lime [*Citrus aurantifolia* (Christm.) Swingle] is a valuable commercial fruit crop grown in Nepal's Terai to high hills which has high economic, cultural and medicinal importance. Due to low quality planting materials and poor orchard management, production and productivity of acid lime are extremely low in Nepal. The present study aimed at optimization of Inter-Simple Sequence Repeat (ISSR)-polymerase chain reaction (PCR) reaction and cycling conditions for PCR amplification and genetic diversity assessment of acid lime cultivars from eastern agro-ecological zone, Nepal. Five different parameters [*viz.* Template DNA, $MgCl_2$, Deoxynucleotide triphosphate (dNTPs), Primers and *Taq* DNA polymerase] were used in the ISSR-PCR reaction optimization. Moreover, 4 different cycling conditions were assessed for the determination of the optimum range for ISSR-PCR profiling. The optimized PCR reaction conditions were found to be 25 ng DNA, 3.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM Primers and 1.5 Unit *Taq* DNA polymerase and best PCR cycling condition consisted of initial denaturation of 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, elongation at 72°C for 2 min and final elongation of 7 min at 72°C. The results from this study were successfully used for ISSR-PCR based genetic diversity assessment of Nepalese acid lime genotypes to find out the elite cultivars of Eastern Nepal.

Key words: Acid lime, genetic diversity, polymerase chain reaction (PCR) optimization.

INTRODUCTION

Citrus L. is a member genus of the Rutaceae family. Most *Citrus* species are found in tropical and subtropical regions of the world (Liu et al., 2012). In the context of Nepal, citrus fruit farming may have begun in ancient times, based on descriptions of the fruits in old scriptures about religious ceremonies and medicinal values (NCRP,

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2012). Although the history of citrus fruit cultivation in Nepal is not well known, commercial citrus fruit cultivation in Nepal began only after 1970 (NCRP, 2012). Citrus fruits are estimated to be grown in 66 of Nepal's 77 districts, accounting for roughly 30% of the total area under fruit cultivation (NCRP, 2012). Citrus fruit is one of Nepal's most important fruits in terms of area covered, yield, and export potential, and the agriculture perspective plan has designated it as a high-value cash crop (APP, 1995). In Nepal, there are 11 lower taxa of *Citrus* and *Citrus aurantifolia* (Christm.) Swingle is one of these species (Press et al., 2000).

C. aurantifolia (Christm.) Swingle is cultivated all over the world (Enejoh et al., 2015) and is known as Indian lime, Lime, Key lime or Acid lime (USDA, 2013). However, it is commonly called as "Kagati" in Nepal. It is one of the economically important and most consumed plant species on the globe (Lu et al., 2006). In 2015, Mexico was the top producer of lime and lemon with 2270, followed by Argentina (1450), EU (1286), and USA (784) in the 1000 metric tons unit (Narang and Jiraungkoorskul, 2016). In Nepal's scenario, acid lime has been cultivated in many districts (60 out of 77) from the foothills of the Himalayas to high hill landscapes (NCRP, 2012). In terms of area coverage after mandarin and sweet orange, acid lime is the third commercially important fruit crop cultivated in Nepal (Munankarmi et al., 2018). The best time to grow acid lime in Nepal is from September to November, but the demand for this fruit is constant throughout the year (Dhakal et al., 2002). However, in terms of acid lime productivity, Nepal produces significantly less (8.3 tons/ha) (MoAC, 2011) than other countries such as Argentina (19 tons/ha) and India (12.2 tons/ha) (FAO, 2006).

Citrus fruits contain important chemical constituents and different secondary metabolites. They are high in citric acid, flavonoids, phenolics, pectins, limonoids, ascorbic acid, and other nutrients (Dugo and Di-Giacomo, 2002). Moreover, glucose, sucrose and malic acid are contained in fruit juice of acid lime (Aubert et al., 1990). Citrus fruits contain carbohydrates, vitamin, acids, nitrogen compounds, enzymes, pigments, lipids, and volatile compounds as major constituents (Shrestha, 1999). Various citrus elements, such as phenolics, carotenoids, limionoids, vitamin C, water soluble B-complex vitamin, specifically folic acid and thiamine (vitamin B1) are currently being researched in terms of health (Lallan and Singh, 2006).

In *Citrus*, there are frequent incidences of hybridization, apomixis, polyploidy and bud mutation (Kumar et al., 2010). This throws the taxonomy of the *Citrus* genus into disarray and complicates genetic diversity assessments (Kumar et al., 2010). Since morphological markers are affected by the environment, they have significant limitations for assessing diversity. Biochemical markers are more effective than morphological markers, but their number is limited for evaluation (Semagn et al., 2006). DNA-based molecular markers, on the other hand, are plentiful, superior, and provide unbiased prediction of genetic diversity and identification of particular sequence in the DNA pool, but each molecular marker has its own set of limitations (Semagn et al., 2006). Continuous infusions of wild relatives, traditional varieties, and the use of modern breeding techniques are needed to boost crop genetic resources. Sometimes an increase in a crop species' yield is followed by a decrease in variability among its cultivated varieties, resulting in genetic erosion, which restricts further improvement in the species (Singh, 2005). The use of genetically suitable diverse and unrelated parents in breeding programs will help to prevent these worst-case scenarios (Singh, 2005).

Traditionally, most *Citrus* breeders have used morpho-physiological traits including color, height, and maturity time to evaluate genetic diversity (Ni et al., 2002). The advancement of DNA marker technology in recent years has developed effective methods for evaluating and measuring genetic diversity and cultivar identification (Ni et al., 2002). Morphological markers have been discovered in nature or as a result of mutagenesis experiments and usually lead to visually score able qualitative traits which are either dominant or recessive in nature (Chawla, 2002). However, in terms of molecular markers, the introduction of Polymerase Chain Reaction (PCR) has changed the vision for studies of hybridization, conservation biology, introgression and phylogenetics (Soltis et al., 1998). Different parameters (primers, deoxynucleotide triphosphate, DNA concentrations, $MgCl_2$ concentrations and polymerase concentration) with different concentrations and ranges are used for the PCR; among which only optimum conditions perform best (Jones, 2002). For the assessment of genetic diversity, a variety of (PCR)-based molecular marker techniques are now available. Rand Amplified Polymorphism DNA (RAPD), Inter-Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR) markers are the most widely used molecular markers in diversity testing (Shrestha et al., 2010a).

ISSRs are a modified form of the SSR method that uses a single primer based on microsatellites found across the genome (Zietkiewicz et al., 1994). ISSR primers can provide accurate and reproducible bands due to their high annealing temperature and longer sequence, and the cost of analysis is cheaper than that of certain other markers, such as AFLP (Guo et al., 2009). Therefore, ISSRs are commonly used to uncover plants' genetic diversity (Qiu et al., 2009). Using a co-dominant SSR marker, the genetic diversity of acid lime was investigated at the molecular level in Nepal's various agro-ecological zones (Shrestha et al., 2012). In this context, assessing acid lime landrace genetic diversity using the dominant ISSR marker will be useful for comparing findings with those obtained using the SSR marker and selecting genotypes for potential breeding

Table 1. Altitudinal range, accession numbers and locality details of acid lime samples.

High-hills			Mid-hills			Terai		
Acc. No.	Altitude (m asl)	VDC-Ward No.	Acc. No.	Altitude (masl)	VDC-Ward No.	Acc. No.	Altitude (masl)	VDC-Ward No.
LT-1	1605	Okhre-8	LD-49	1185	Bodhe-1	LM-43	135	Sunpur-2
LT-17	1750	Fachmara-7	LKv-60	1285	Balara-1	LM-44	135	Sunpur-2
LT-18	1710	Fachmara-9	LKm-61	1285	Balara-1	LD-45	135	Sunpur-2
LT-15	1655	Fachmara-9	LKr-62	1285	Balara-1	LD-58	135	Sunpur-2
LD-50	1638	Rajarani-9	LD-48	1181	Bodhe-1	LS-34	128	Narsing-2
LT-8	1505	Okhre-8	LD-25	1180	Balara-1	LS-35	128	Narsing-4
LT-22	1505	Sudap-1	LD-26	1175	Balara-1	LS-36	128	Narsing-4
LT-9	1500	Okhre-5	LD-27	1175	Balara-1	LS-37	128	Narsing-4
LT-21	1485	Fachamara-1	LD-28	1175	Balara-1	LS-38	128	Narsing-4
LT-20	1410	Fachamara-8	LD-29	1175	Balara-1	LS-39	128	Narsing-4
LT-16	1405	Fachamara-7	LD-30	1175	Balara-1	LS-40	128	Narsing-4
LT-19	1350	Fachamara-7	LD-59	1175	Balara-1	LS-41	128	Narsing-4
LT-13	1315	Fachamara-7	LT-4	1155	Okhre-1	LS-42	128	Narsing-4
LT-12	1310	Fachamara-7	LT-5	1155	Okhre-3	LS-56	128	Narsing-4
LT-14	1308	Fachamara-7	LT-6	1150	Okhre-3	LS-57	128	Narsing-4
LT-23	1308	Sudap-7	LD-31	1150	Dhnk -3	LM-51	125	Pathari-2
LT-3	1305	Okhre-8	LT-7	1145	Okhre-2	LM-52	125	Pathari-2
LD-24	1290	Balehara-8	LT-10	1135	Okhre-3	-	-	-
LT-2	1285	Okhre-1	LT-11	1130	Okhre-3	LM-54	125	Pathari-2
LD-46	1278	Bodhe-2	LD-32	1130	Balhra-3	LM-55	125	Pathari-2
-			LD-33	1130	Balhra-1	-	-	-

LT, Lime Terhathum district; LD, Lime Dhankuta district; LM, Lime Morang district; LS, Lime Sunsari district; LKm, Lime madras; LKr, Lime Rampur; LKv, Lime Varanasi; VDC = Village Development Committee, masl = meter above sea level.
Source: Munankarmi et al. (2018).

programs. Furthermore, in our previous study (Munankarmi et al., 2018), the findings from this research were used to generate reliable genetic profiles for the assessment of genetic diversity among acid lime cultivars of eastern Nepal.

MATERIALS AND METHODS

Plant Materials

Sixty young acid lime leaf samples (6 to 8 weeks old) were collected and preserved in silica gel in air tight zip-lock bags from farmers' plantation areas of Eastern Nepal for subsequent experiments. All agro-ecological areas, namely Terai (below 600 m asl), Mid-hills (600-1200 m asl), and High-hills (above 1200 m asl) were sampled at random from selected trees (Munankarmi et al., 2018) (Table 1).

DNA extraction

The DNA extraction of acid lime samples was performed using DNeasy mini kits (Qiagen Ltd, Crawley, UK) according to the Qiagen catalogue provided.

DNA quantification and testing for purity

The yield of DNA extracted from leaf tissue was measured using spectrophotometric method (Biophotometer, Eppendrof-AG22331, Germany). Each DNA sample was quantified for its concentration along with its purity assessment using the Biophotometer.

Gel electrophoresis

The extracted DNA was analyzed on a 1.5% Agarose gel in TAE buffer (1X) at 50 V (8.33 V/cm) for 0.5 h, while ISSR-PCR amplified products were analyzed on a 2% Agarose gel at 50 V (8.33 V/cm) for 2 h and SSR-PCR amplified products on a 4% Agarose gel at 70 V (11.67 V/cm) for 1.5 h using Major Science gel tank (volume loaded in the well was 4 µl DNA and 1 µl GLB (6X) for DNA analysis and 10 µl PCR product and 2 µl GLB (6X) for product analysis. The Ethidium Bromide (EtBr) (10 mg/ml; Promega) was added during gel preparation at a concentration of 5 µl/100 ml gel. After the gel run, the gels were visualized on a gel documentation system (IN GENIUS, Syngene Bio-imaging, UK).

PCR Optimization and primer screening

All PCR experiments were performed in a thermal cycler (BIOER, China). Cycling conditions (Fang et al., 1998; Shahsavar et al., 2007; Kumar et al., 2010; Lui et al., 2011) were accessed for PCR

Table 2. Tested different ISSR PCR-cycling conditions.

Author	Cycling conditions		
Fang et al. (1998)	Initial Denaturation	94°C for 4 min	
	Denaturation	94°C for 30 s	27 cycles
	Annealing	52°C for 45 s	
	Extension (Elongation)	72°C for 2 min	
	Final extension	72°C for 7 min	
	Hold	4°C for 4 min	
Kumar et al. (2010)	Initial Denaturation	94°C for 4 min	
	Denaturation	94°C for 1 min	35 cycles
	Annealing	52°C for 1 min	
	Extension (Elongation)	72°C for 2 min	
	Final Extension	72°C for 7 min	
	Hold	4°C for 4 min	
Shahsavar et al. (2007)	Initial Denaturation	94°C for 5 min	
	Denaturation	94°C for 30 s	40 cycles
	Annealing	50°C for 45 s	
	Extension (Elongation)	72°C for 2 min	
	Final Extension	72°C for 7 min	
	Hold	4^0C for 5 min	
Lui et al. (2011)	Initial Denaturation	94°C for 5 min	
	Denaturation	94°C for 30 s	35 cycles
	Annealing	52°C for 45 s	
	Extension (Elongation)	72°C for 2 min	
	Final Extension	72°C for 5 min	
	Hold	4°C for 4 min	

Table 3. Parameters selected and tested for PCR optimization.

PCR Parameter	Tested range
DNA concentration (ng)	1.2, 25.0, 37.5, 50.0, 62.5, 75.0, 87.5 and 100.0
$MgCl_2$ concentration (mM)	1.5, 2.0, 2.5, 3.0, 3.5 and 4.0
dNTPs (mM)	0.1, 0.2, 0.3, 0.4 and 0.5
Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6
Taq DNA polymerase (Unit)	0.5, 1.0, 1.5, 2.0 and 2.5

optimization (Table 2). PCR reaction conditions were optimized by varying several PCR parameters. These parameters include: PCR cycling conditions, DNA, $MgCl_2$, Primer, Deoxynucleotide triphosphate (dNTPs) and *Taq* DNA polymerase concentrations. A range of DNA concentrations (1.2, 25.0, 37.5, 50.0, 62.5, 75.0, 87.5 and 100.0 ng) of sample was assessed. Similarly, different $MgCl_2$ concentrations (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM), Primer concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 µM), dNTPs concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mM) and *Taq* polymerase concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 unit) were assessed for the selection of best and optimum ISSR-PCR reaction mixture (Table 3). For controlling false positive results, a negative control reaction was run in each optimization experiment. Altogether, 49 ISSR primers were used for the screening against acid lime LS15 (Table 4).

RESULTS

Quantification and purity testing of DNA

Quantification of DNA was done on a Bio-photometer (Eppendorff, Germany). The DNA concentrations of acid lime landraces extracted with the Qiagen kit ranged from

Table 4. Primers for the screening against acid lime LS15.

Primer code	Primer sequence (5' - 3')	Primer length	GC content (%)
C1	TCTCTCTCTCTCTCTCCC	20 mer	55
C2	AGCAGCAGCAGCGT	14 mer	64.3
C4	CTCCTCCTCGC	11 mer	72.7
C5	CACCACCACGC	11 mer	72.7
C7	HVHGAGAGAGAGAGAGAT	18 mer	47.2
C8	TCCTCCTCCTCCTCCRY	17 mer	64.7
C9	BDBTCCTCCTCCTCCTCC	18 mer	63.9
C10	HVHTCCTCCTCCTCCTCC	18 mer	63.9
UBC 807	AGAGAGAGAGAGAGAGT	17 mer	47.1
UBC 809	AGAGAGAGAGAGAGAGG	17 mer	52.9
UBC 810	GAGAGAGAGAGAGAGAT	17 mer	47.1
UBC 811	GAGAGAGAGAGAGAGAC	17 mer	52.9
UBC 812	GAGAGAGAGAGAGAGAA	17 mer	47.1
UBC 817	CACACACACACACACAA	17 mer	47.1
UBC 818	CACACACACACACACAG	17 mer	52.9
UBC 819	GTGTGTGTGTGTGTGTA	17 mer	47.1
UBC 820	GTGTGTGTGTGTGTGTC	17 mer	52.9
UBC 821	GTGTGTGTGTGTGTGTT	17 mer	52.9
UBC 822	TCTCTCTCTCTCTCTCA	17 mer	47.1
UBC 824	TCTCTCTCTCTCTCTCG	17 mer	52.9
UBC 825	ACACACACACACACACT	17 mer	47.1
UBC 826	ACACACACACACACACC	17 mer	52.9
UBC 830	TGTGTGTGTGTGTGTGG	17 mer	52.9
UBC 834	AGAGAGAGAGAGAGAGYT	18 mer	47.2
UBC 835	AGAGAGAGAGAGAGAGYC	18 mer	52.8
UBC 836	AGAGAGAGAGAGAGAGYA	18 mer	47.2
UBC 840	GAGAGAGAGAGAGAGAYT	18 mer	47.2
UBC 841	GAGAGAGAGAGAGAGAYC	18 mer	52.8
UBC 842	GAGAGAGAGAGAGAGAYG	18 mer	52.8
UBC 844	CTCTCTCTCTCTCTCTAGC	19 mer	52.6
UBC 845	CTCTCTCTCTCTCTCTAGG	19 mer	52.6
UBC 848	CACACACACACACACARG	18 mer	52.8
UBC 850	GTGTGTGTGTGTGTGTYC	18 mer	52.8
UBC 853	CTCTCTCTCTCTCTCTAGT	19 mer	47.4
UBC 856	ACACACACACACACACYA	18 mer	47.2
UBC 857	ACACACACACACACACYG	18 mer	52.8
UBC 860	TGTGTGTGTGTGTGTGRA	18 mer	47.2
UBC 864	ATGATGATGATGATGATG	18 mer	33.3
UBC 867	GGCGGCGGCGGCGGCGGC	18 mer	100
UBC 868	GAAGAAGAAGAAGAAGAA	18 mer	33.3
UBC 873	GACAGACAGACAGACA	16 mer	50.0
UBC 880	GAAGAGGAGAGGAGA	15 mer	60.0
UBC 886	VDVCTCTCTCTCTCTCT	17 mer	50.0
UBC 888	BDBCACACACACAGACA	17 mer	50.0
UBC 889	DBDACACACACACACACA	18 mer	47.2
UBC 890	VHVGTGTGTGTGTGTGT	17 mer	50.0
UBC 891	HVHTGTGTGTGTGTGTG	17 mer	50.0
UBC 895	AGAGTTGGTAGCTCTTGATC	20 mer	45.0
UBC 900	ACTTCCCCACAGGTTAACACA	21 mer	47.6

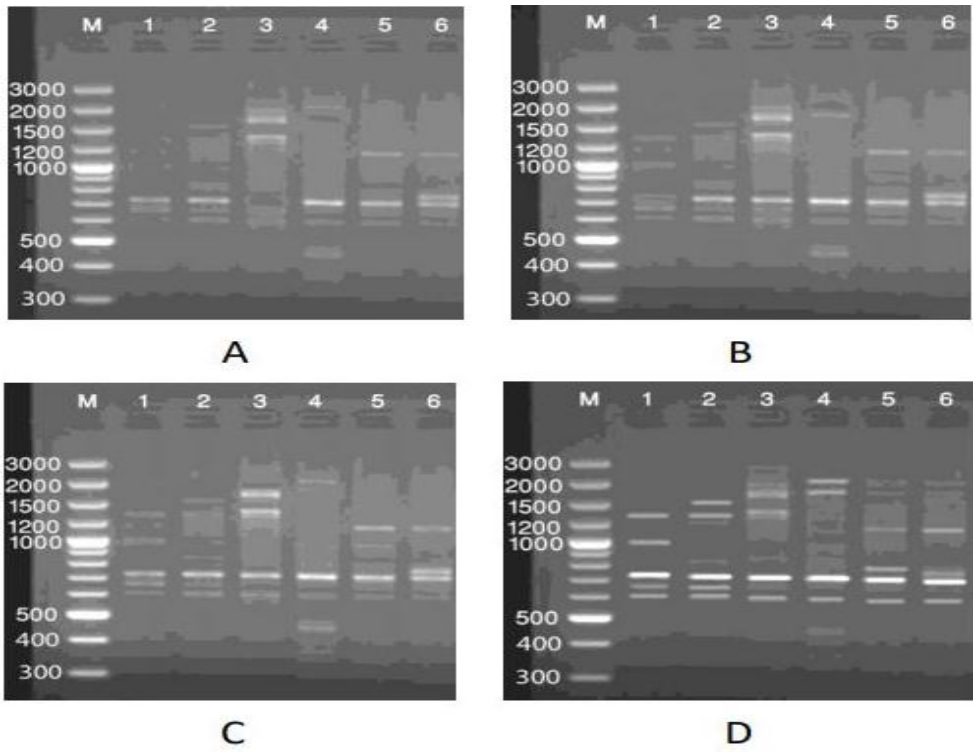

Figure 1. Gel Picture of ISSR-PCR for the selection of best cycling condition using primer C4 and the genomic DNA of samples LT-1, LT-2, LT-3,LT- 4, LT-5 and LT-6 in lanes 1-6 respectively. Lane 'M' are 100 bp plus molecular weight marker; A = Cycling condition as of Fang et al. (1998); B = Cycling condition as of Lui et al. (2011); C = Cycling condition as of Kumar et al. (2010) and D = Cycling condition as of Shahsavar et al. (2007).

Table 5. Selection of optimized parameters for ISSR-PCR among different ranges and concentrations.

PCR parameter	Optimum condition
DNA concentration (ng)	25
$MgCl_2$ concentration (mM)	3.0
dNTPs (mM)	0.4
primer concentration (µM)	0.4
Taq DNA polymerase (Unit)	1.5

4 µg/ml to 126.8 µg/ml, with a purity range of 0.5 to 2.13.

ISSR-PCR cycling condition optimization

Among the four cycling conditions assessed, the PCR program (cycling condition) described by Shahsavar et al. (2007) with initial denaturation of 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, elongation at 72°C for 2 min and final elongation of 7 min at 72°C was found to be the best for ISSR profiling of Nepalese *C. aurantifolia* landraces and hence selected for subsequent ISSR profiling experiments (Figure 1).

ISSR-PCR reaction condition optimization

Among the tested DNA concentration range, optimized concentration was found using 25 ng (Table 5). Most of the concentration produced the reproducible bands, but 25 ng of template DNA was selected as the optimum condition since it is the minimum concentration that produces discernible bands (Figure 2). Moreover, crispy bands observed at 3.0 mM $MgCl_2$ were selected as optimum concentration for subsequent experiments as it

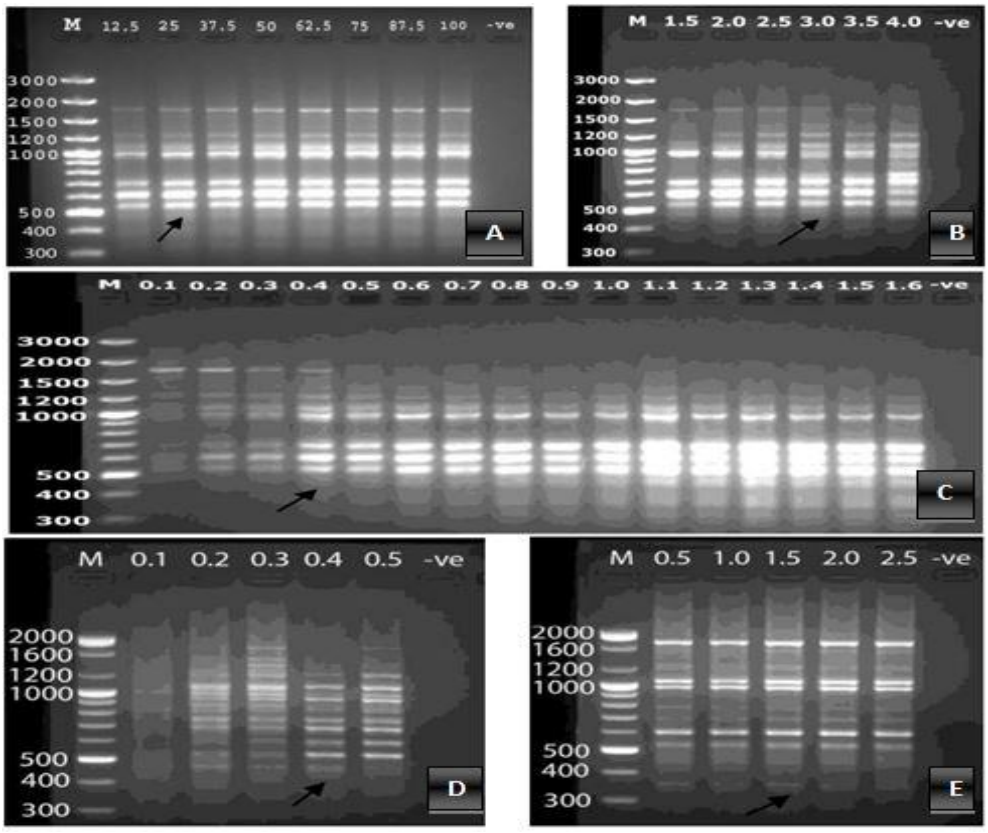

Figure 2. Gel pictures of ISSR-PCR for the optimal condition selection (A) Each lane is marked with the respective concentrations of template DNA (12.5 to 100 ng) (B) Each lane is marked with the respective concentrations of $MgCl_2$ (1.5 to 4.0 mM) used during ISSR-PCR (C) Each lane is marked with the respective concentrations of primer UBC 809 (0.1 to 1.6 µM) used during ISSR-PCR (D) Each lane is marked with the respective concentrations of dNTPs (0.1 to 0.5 mM) (E) Each lane is marked with the respective concentrations of *Taq* polymerase (0.5 to 2.5 U) used during ISSR-PCR. An arrow in each figure determines the optimal condition opted.

revealed a number of bands with a clear and crispy nature (Figure 2). Among 16 primer concentrations, the banding pattern suggested primer concentration ranging from 0.2 to 1.6 µM to be good. However, 0.4 µM concentration was selected for subsequent ISSR-PCR profiling experiments as it produced clear and crispy bands (Figure 2). Observed ISSR patterns for dNTPs revealed that concentration of 0.4 mM produced crispy bands and selected as optimum concentration (Figure 2). Though all the *Taq* DNA polymerase concentration used in the experiment produced ISSR bands, the best banding pattern was observed for 1.5Units for 1.5U (Figure 2).

Primer screening for ISSR profiling

The optimized ISSR-PCR reactions and cycling conditions were used for screening of 49 different ISSR primers using the fresh genomic DNA of acid lime (*C. aurantifolia*) sample LS-15. Out of 49 primers, 21 primers (Figure 3) which gave multiple, scorable, crispy and reproducible bands were selected finally to be used in ISSR-PCR based genetic diversity analysis involving 60 accessions of *C. aurantifolia* (acid lime) under study. The experiments were repeated twice for the conformation of reproducibility of ISSR amplifications.

ISSR profiling of acid lime

The ISSR-PCR profiles generated by the selected 21 primers were used for genetic diversity assessment of *C. aurantifolia* (acid lime) landraces under study. Representative ISSR profiles generated by primers C8 and UBC842 are as shown in Figures 4 and 5, respectively. Across the 60 acid lime accessions, a total of 234 loci were amplified, with an average of 9.72 bands per primer. Eight of the 21 primers showed 100% polymorphisms. The number of scorable bands created per primer varied between 7 and 18, with amplicon sizes ranging from 250 to 3200 bp. In the total accessions

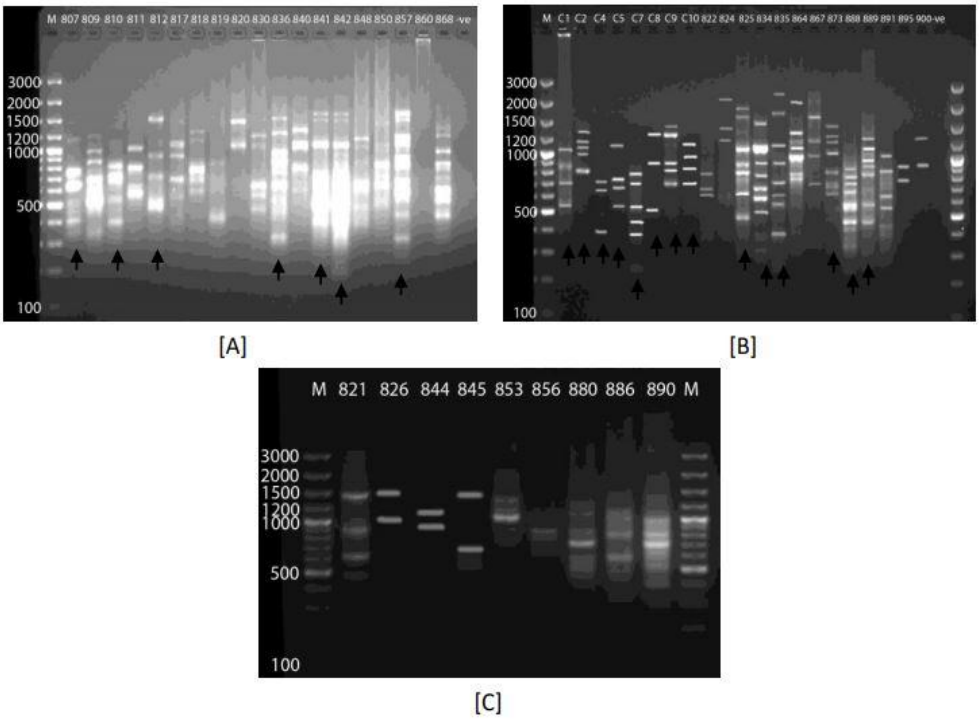

Figure 3. ISSR gel pictures for the Primer Screening using template DNA of Sample LS-15. Each lane in [A] [B] and [C] corresponds to all 49 primers used in the experiment. Lanes marked 'M' are 100 bp plus DNA ladder. Arrows in the figures indicated the selected one.

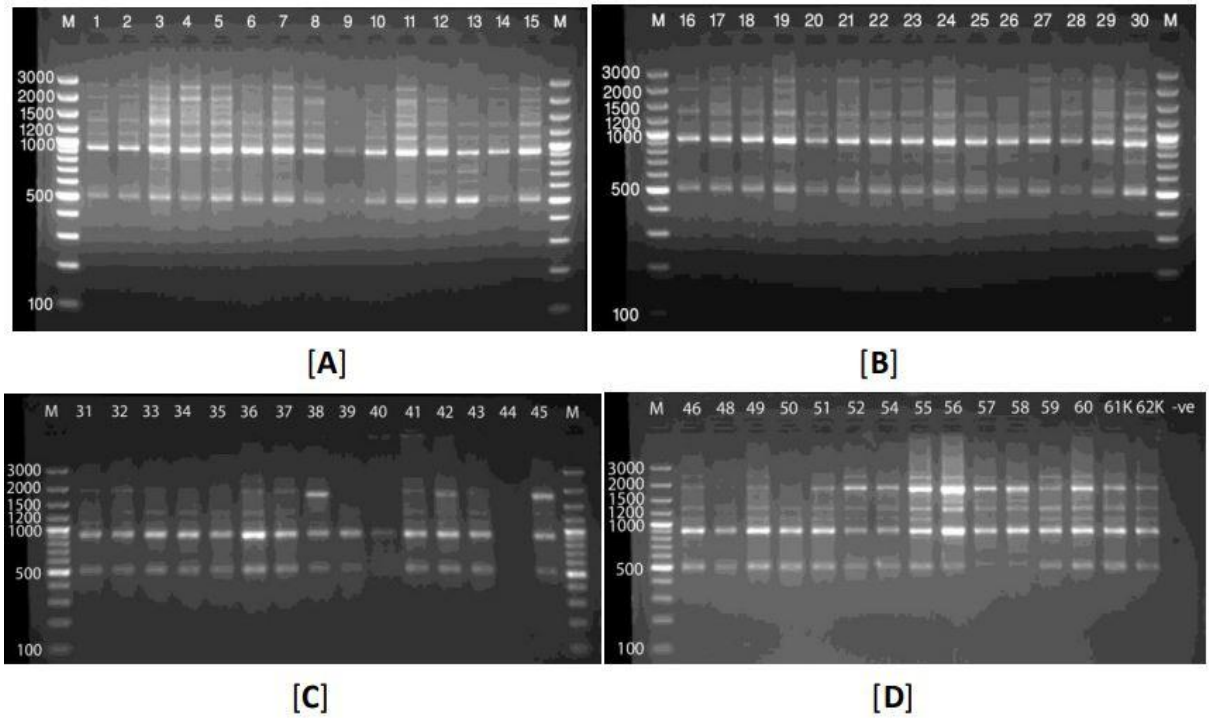

Figure 4. ISSR profiles amplified with Primer C8. Lanes marked with M are 100 bp plus molecular weight markers. [A] Lanes 1-15 represent acid lime samples 1-15; [B] Lanes 16-30 represent acid lime samples 16-30; [C] Lanes 31-45 represent acid lime samples 31-45; [D] Lanes 46-62k represent acid lime samples 46-62k.

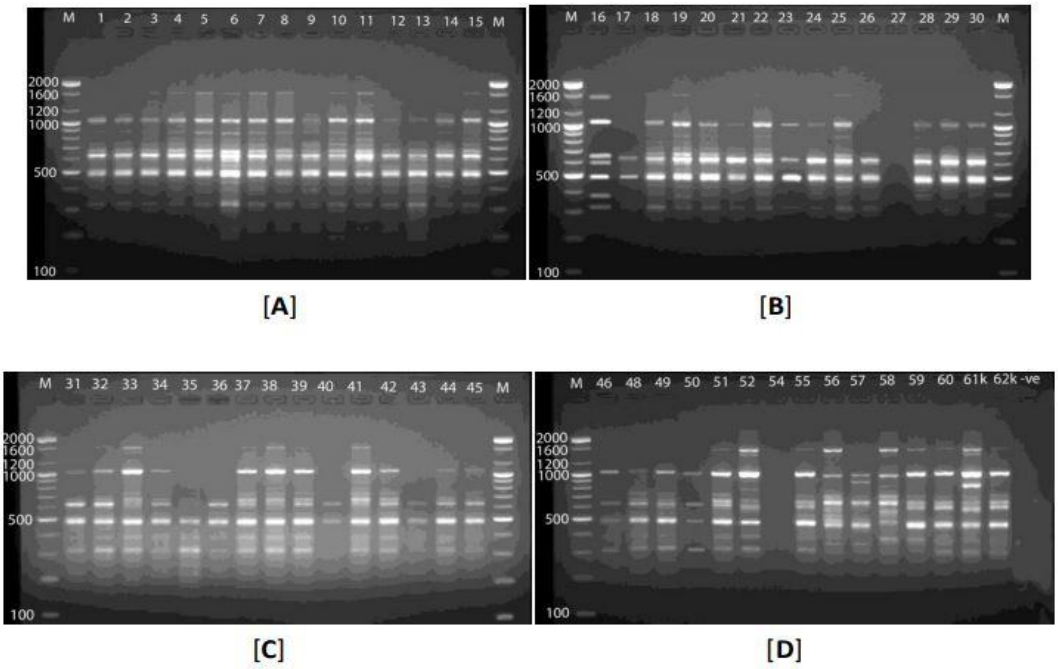

Figure 5. ISSR profiles amplified with Primer UBC 842. Lanes marked with M are 100 bp plus molecular weight markers. [A] Lanes 1-15 represent acid lime samples 1-15; [B] Lanes 16-30 represent acid lime samples 16-30; [C] Lanes 31-45 represent acid lime samples 31-45; [D] Lanes 46-62k represents acid lime samples 46-62k.

checked, primer UBC857 produced the highest number of ISSR loci (18), while primer C1 and UBC834 produced the lowest number of ISSR loci (7) (Table 6).

DISCUSSION

PCR is a highly sensitive assay that can amplify a single DNA molecule from complex mixtures of genomic sequences. Since no single PCR amplification protocol would be suitable for all cases, each new PCR application will need optimization. The existence of non-specific background bands due to mis-priming or mis-extension of the primers, the development of "primer-dimers" that compete for amplification with the desired product, and mutations or heterogeneity due to mis-incorporation are just a few of the issues that often arise in such studies (Saiki et al., 1985; Mullis and Faloona, 1987). In the present investigation, ISSR-PCR reaction and cycling conditions for acid lime have been optimized. These optimized conditions were then subsequently used for the profiling of various acid lime samples by ISSR primers under study.

Quantification and purity testing of DNA

The DNA concentrations of acid lime landraces extracted with the Qiagen kit ranged from 4 µg/ml to 126.8 µg/ml, with a purity range of 0.5 to 2.13. A higher absorbance value of 2.0 suggested the existence of phenol contamination in extracted DNA, while a lower value indicated the presence of proteins (Subedee et al., 2020).

ISSR-PCR cycling condition optimization

PCR cycling parameters like temperatures, duration and ramping rate during denaturation, annealing, extension step and number of amplification cycles play significant roles for optimal banding patterns (Weising et al., 2005; Shrestha et al., 2010b). The most likely cause for failure of a PCR is incomplete denaturation of the target template and/or the PCR product. An applicable annealing temperature is 5°C below the true melting temperature (Tm) of the primers (Innis et al., 1988; Innis and Gelfand, 1990). The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized. Too many cycles can increase the amount and complexity of non-specific background products (Plateau Effect) and too few cycles give low product yield (Innis and Gelfand, 1990).

Out of 4 randomly selected ISSR-PCR programs, cycling conditions described by Shahsavar et al. (2007) produced the best banding pattern for ISSR profiling of acid lime DNA in the present investigation. Though other programs also give appreciable banding patterns, the

Table 6. Primer sequences, total number of amplified band (TNB), number of polymorphic bands (NPB), percent polymorphism and amplicon size range of the 21 primers used to generate ISSR-PCR profiles in 60 *Citrus aurantifolia* (Acid lime) accessions.

Primer Code	Primer sequence (5' - 3')	Primer length	TNB	NPB	Polymorphisms (%)	Amplicon size range (bp)
C1	TCTCTCTCTCTCTCTCCC	20 mer	**7**	5	71.43	550-1800
C2	AGCAGCAGCAGCGT	14 mer	10	10	100	500-3200
C4	CTCCTCCTCGC	11 mer	14	14	100	300-2200
C5	CACCACCACGC	11 mer	12	11	91.67	600-2500
C7	HVHGAGAGAGAGAGAGAT	18 mer	17	15	88.23	250-2000
C8	TCCTCCTCCTCCTCCRY	17 mer	9	7	77.78	520-2800
C9	BDBTCCTCCTCCTCCTCC	18 mer	9	6	66.67	520-2000
C10	HVHTCCTCCTCCTCCTCC	18 mer	11	8	72.73	500-2000
UBC 807	AGAGAGAGAGAGAGAGT	17 mer	9	9	100	450-1300
UBC 810	GAGAGAGAGAGAGAGAT	17 mer	16	16	100	390-1980
UBC 812	GAGAGAGAGAGAGAGAA	17 mer	12	9	75	450-1500
UBC 825	ACACACACACACACACT	17 mer	9	5	**55.56**	500-2000
UBC 834	AGAGAGAGAGAGAGAGYT	18 mer	**7**	5	71.43	310-1550
UBC 835	AGAGAGAGAGAGAGAGYC	18 mer	10	10	100	400-2900
UBC 836	AGAGAGAGAGAGAGAGYA	18 mer	10	10	100	320-1450
UBC 841	GAGAGAGAGAGAGAGAYC	18 mer	10	10	100	320-1700
UBC 842	GAGAGAGAGAGAGAGAYG	18 mer	12	12	100	320-1650
UBC 857	ACACACACACACACACYG	18 mer	**18**	17	94.44	300-3000
UBC 873	GACAGACAGACAGACA	16 mer	14	13	92.86	470-3000
UBC 888	BDBCACACACACAGACA	17 mer	9	7	77.78	480-1450
UBC 889	DBDACACACACACACACA	18 mer	9	5	55.56	480-1400
Total	-	-	234	204	-	-

Average polymorphic bands per primer = 9.72; average polymorphism = 87.18.

best, reproducible, crispy and clear bands were given by the program described in the aforementioned paper. This may be because the number of cycles in this program is high, that is, 40 cycles in comparison to others and low annealing temperature, that is, 50°C. During the present investigation, very few non-reproducibility cases were encountered. This reproducibility was checked by repeating the doubtful experiments.

ISSR-PCR reaction condition optimization

In order to maintain reproducible banding patterns among the laboratories, optimization of various PCR reaction parameters is necessary (Shrestha, 2001; Weising et al., 2005). The following sensitive reaction parameters were critically optimized for the development of the standard ISSR-PCR protocol for acid lime: template DNA, MgCl2, Primer, dNTPs, and *Taq* DNA polymerase concentrations. The minimum concentration of template DNA that gives the best crispy banding patterns was observed at 25 ng. Templates may have a considerable influence on the results of a PCR, and their quality and quantity were the main factors affecting reproducibility (Weising et al., 2005). For most of the species, good results have been achieved for concentrations ranging from 50 to 100 ng in 25 to 50 µl PCR reaction volume (Shrestha, 2001; Padmalatha and Prasad, 2006). So, in the present investigation, DNA concentrations ranging from 12.5 to 100 ng in 25 µl reaction volume were tested. Negative control (without template DNA) was included and no bands were observed indicating free of false positive results. A high concentration of DNA usually inhibits PCR-amplification, making primer annealing difficult (Micheli et al., 1997), so a low concentration was used.

In the present study, a 3.0 mM concentration of $MgCl_2$ was considered as optimum for the PCR amplification. $MgCl_2$ is a cofactor of the *Taq* polymerase enzyme and its concentration affects primer annealing, strand dissociation temperatures of template and PCR product, product specificity, and formation of primer-dimer artifacts, enzyme activity and fidelity (Innis and Gelfand, 1990). Generally, increasing amounts of Mg^{++} results in the accumulation of non-specific amplification products, and insufficient Mg^{++} will reduce the yield (Wiliams et al., 1991). Though strong and reproducible bands are obtained over a wide range of Mg^{++} concentration, a change in concentration often results in a qualitative change of fragment patterns (Wiliams et al., 1993). The $MgCl_2$ can be optimized either by determining the optimal

primer/template combination (primer screening) in the fixed $MgCl_2$ concentration or varying $MgCl_2$ concentration for fixed primer/template combination (Shrestha, 2001). In the present investigation, the latter strategy was followed.

Tested ranges of primer concentration were 0.1 to 1.6 µM. The banding pattern at 0.4 µM, which is minimum concentration, was crispy and reveled more number of amplified bands for ISSR, so selected as optimum concentration and used for further PCR reactions. Primer concentration plays significant role in the outcome of PCR. High concentration of primer promotes mis-priming, accumulation of non-specific products and formation of primer dimer where as low concentration leads to amplification failure. The primer dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs, and primers, resulting in a lower yield of the desired product (Innis and Gelfand, 1990; Padmalatha and Prasad, 2006).

In the present investigation, dNTPs were tested in the range of 0.1 to 0.5 mM concentration, among which the concentration at 0.4 mM gave clear and crispy bands for ISSR-PCR. Concentrations of dNTP were reported to have an effect on the pattern of DNA amplification. Both the specificity and the fidelity of PCR are increased by using lower dNTP concentrations. Low dNTP concentrations minimize mis-priming at non-target sites and reduce the likelihood of extending mis-incorporated nucleotides (Innis et al., 1988). Magnesium is known to be chelated by dNTPs, and high concentrations of dNTPs increase the error rate of *Taq* polymerase, interfering with its activity (due to Mg++'s limited freedom) (Padmalatha and Prasad, 2006).

For the present experiment, *Taq* DNA polymerase concentration of 1.5 U was selected as optimum for further ISSR experiment. A recommended concentration range for *Taq* DNA polymerase is between 1 and 2.5 units per 100 µl reaction when other parameters are optimum (Lawyer et al., 1989). *Taq* DNA polymerase from different suppliers may behave differently because of different formulations, assay conditions, and/or unit definitions. If the enzyme concentration is too high, non-specific background products may accumulate, and if too low, an insufficient amount of desired product is made (Innis and Gelfand, 1990; Weising et al., 2005). However, the choice of enzyme depends on the requirements of the PCR experiment, especially with respect to specificity, efficiency or fidelity (Cha and Thilly, 1993).

Primer screening for ISSR profiling

The sufficiency of differentiating different genotypes in PCR-based methods varies greatly between primers. Usually, it is advisable to screen a considerable number of primers on a few samples in order to find an optimal set, which can then be used for the entire materials under study (Weising et al., 2005). In the present ISSR-PCR profiling, 49 primers were screened using fresh genomic DNA of LS15 acid lime (*C. aurantifolia*) sample, based on number of fragments (bands) and their visibility using optimized ISSR-PCR reaction and cycling condition. Twenty one were selected on the basis of reproducibility and crispy nature of bands amplified in ISSR-PCR analysis involving all 60 acid lime accessions. The primers having 18 to 28 nucleotides in length and 50 to 60% G+C composition are considered as efficient primers (Innis and Gelfand, 1990). The ISSR primers are usually 16 to 25 bp long, and hence have high reproducibility in comparison to RAPD primers which permits the subsequent use of high annealing temperature (45 - 60°C) leading to higher stringency (Reddy et al., 2002). Further, some of the primers used in this investigation are anchored at 5′ or 3′ end, which might be the reason for high fidelity and reproducibility of the banding patterns. In the past, the number of primers used for genetic diversity study in *Citrus* spp. varied according to different investigators. For example, out of screened 105 ISSR markers, 15 primers which gave best amplification results were selected for polymorphism analysis of Indian wild orange and related wild species (Kumar et al., 2010). Similarly, 6 out of 10 ISSR primers amplified reproducible and polymorphic bands in phenetic analysis between *C. indica* and few commercially important *Citrus* spp. (Marak and Laskar, 2010).

ISSR profiling of acid lime

Molecular markers are imperative in the assessment of genetic diversity, ranging from nucleotide level (SNPs) to gene and allele frequencies (genotype information), and devising various germplasm conservation programs (Sarwat, 2012). The amplified fragments using different primers ranged from 250 to 3200 bp, which is comparable as suggested by Reddy et al. (2002). The banding patterns are qualitative and are rarely informative on their own, so they are evaluated statistically (that is, for translation into biological meaning). The banding patterns originating from different samples have to be compared to each other and for this, individual bands within a lane are assigned to particular positions (molecular weight marker-assisted sizing), and the different lanes are screened for comigrating (that is, matching) bands (Weising et al., 2005). Scoring of ISSR loci was done visually corresponding to an individual for each ISSR primer assessed. The binary data matrix was generated on the basis of presence (1), absence (0) and missing (9) bands and analyzed using appropriate statistical software as published in our previous paper (Munankarmi et al., 2018).

Conclusions

Generation of positive ISSR-PCR gels with solid and

crispy bands are crucial for genetic diversity assessment within and between populations of acid lime landraces. Hence, from this investigation, optimized ISSR-PCR reaction and cycling parameters for acid lime genetic diversity assessment have been obtained. These findings have been utilized to determine an ISSR-PCR based genetic diversity evaluation of Nepalese acid lime genotypes. Genetic diversity analysis using the ISSR-PCR protocol can be utilized in future breeding programme of Acid lime in Nepal.

Author's contributions

NNM and SS designed the experiment. NNM, NR, and SS performed the experiment. SS, TB and NR conceived and supervised the project, NNM, RLS, and SS performed the data analysis. NNM and SC prepared the manuscript.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular characterization of *Escherichia* co-resistance genes from chicken meat

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***Escherichia coli* multi-resistance to a variety of antimicrobials is a result of gene mutation on plasmids, integrons and transposons. The aims of this work were to: (1) detect genotype and phenotype antibiotic resistance genes in *E. coli*, and (2) determine whole-genome sequencing to discover *E. coli* gene multi-drug resistance in chicken meat. Samples were gathered, processed, and analysed bacteriologically; thereafter an antimicrobial sensitivity test was performed and *E. coli* isolates were identified serologically. Results of *E. coli* were 40% from 100 chicken samples. The most potent antibiotics against *E. coli* were Cephalosporins, Quinolones and Oxytetracycline. The serological investigation was as follows: 30% (O157:H7) of STEC, 30% (O142) of ETEC, 10% (O26:H11) of EHEC and 10% EPEC. Subunit B of Shiga-like toxin (SLT) gene showed a symmetrical band, while, Heat-labile toxin (LT) gene was estimated in both plasmid preps in addition to DNA genomic strains. STEC is hazardous to the chicken meat consumers. The study recommended necessary improvement in the hygienic procedures during all processing steps, and minimized the non-important usage of antibiotics to prevent antibiotics resistant.**

Key words: Integrons, Shiga toxin-producing Escherichia coli, Gentamicin, heat-labile toxin, plasmids.

INTRODUCTION

Even though *Escherichia coli* is a nonpathogenic gram-negative intestinal microorganism, it is considered a commensal for both humans and animals. Enteropathogenic, Enteroinvasive, Enterotoxigenic and Enterohaemorrhagic are the four classifications of *E. coli*. However, infectious Shiga toxin-producing *E. coli* (STEC) can cause gastrointestinal disorders such as hemorrhagic colitis (HC), diarrhea and (HUS) haemolytic-uraemic syndrome by drinking contaminated water or eating infected food (Adeyanju and Ishola, 2014).

STEC is the more commonly investigated and serious pathogenic microorganisms that infect humans through poultry meat and are found after drinking contaminated water, dirty food, etc. Whereas beef, chicken and mutton are commonly contaminated by the fecal gut contents of the chicken itself during processing such as slaughter, or by consumers' handlers during processing and storage (EL-Kholy et al., 2020).

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E. coli infections are commonly treated by antibiotics. This results in presence of antibiotic resistant *E. coli* strain, making the microbe multidrug resistant to many antibacterial agents. Microorganisms have antibacterial resistance mainly due to gene mutation, and resistance genes exist in transposons, plasmids and integrons.

An integron is a two component gene capture and dissemination system, initially discovered in relation to antibiotic resistance, and which is found in plasmids, chromosomes and transposons. Integron Class I consists of: Two conversational segments (CS) (CDC, 2019) that are adjacent to the variable region (VR) and are common to the new generation of Enterobacteriaceae. Antibacterial resistance is due to frequent use of antibiotics resulting in a mutated generation of microbial strains, which increases resistant against various antibiotics. It is considered one of the most important public health crises in the world. Scientists are searching for a novel generation of drugs, which are more effective than the currently used resistant-type antibiotics that are used to treat diseased human, poultry and animals (Cunrath et al., 2019).

The purpose of the research is to 1) estimate genotype and phenotype of resistance antibiotic in *E. coli* and 2) identify the microbial resistance gene structure in whole-genome sequencing against poly resistant *E. coli* in commercially available poultry meat.

MATERIALS AND METHODS

Sampling, preparation, bacterial testing

Approximately 100 chicken samples tested were randomly picked from various markets, stored in polyethylene bags, and then immediately transferred to a refrigerator bacteriological laboratory for analysis. Two grams of homogenized chicken meat sample was cultured in MacConkey broth and then incubated for 18 h at 37°C. Next, it was streaked into a MacConkey agar medium (Oxoid) plate at approximately 24 h/37°C. The pink colonies were drawn on eosin methylene blue (EMB) (Oxoid) media for approximately 24 h/37°C. The morphology of *E. coli* appeared as large colonies with a blue-black-green metallic luster. *E. coli* colonies were identified by morphological, microscopic, and biochemical test kits (BioMerieux API, France) (CDC, 2020). Serotyping was used for further identification; and according to WHO, an antiserum set (Denka Seiken Co., Japan) is used (Ewing, 1986).

Antibacterial susceptibility test

Mueller-Hinton agar disc diffusion technology was used consisting of 12 antibiotic discs (30 µg/disc) with the following antibiotics: Gentamicin, Streptomycin, Ampicillin, Penicillin, Cefepim, Cefotaxim, Ciprofloxacin, Flumequine, Trimethophrim, Sulfametoxazole, Tetracycline and Doxycycline (Alderman and Smith, 2001).

Serological identification of *E. coli* by slide agglutination test

The test involved polyvalent and monovalent *E. coli* standard anti-serum (Li et al., 2013) that defines the enteric pathogenic type as follows: Emulsified preparation of microbial colonies was applied as two drops on a glass slide. Looped antiserum was added, aggregated, and another colony was cultivated on nutrient gradient agar and then incubated at 37°C for 24 h to test monovalent serum. A suspension of microorganisms in physiological saline was prepared and a slide agglutination test was performed to identify the antigen.

Nucleic acid extraction

DNA was extracted with the GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, USA). In summary, the microbial colonies were centrifuged for 10 min; 5000 µg of the cells pellet was resuspended in 180 µl solution for digestion (included in the kit), 20 µl K Proteinase was then added, mixed well, and incubated in a water bath at 56°C for about 30 min while shaking continuously until dissolution was complete. Further, vortex 20 µl RNase solution was added to the mixture and incubated for 10 min at 37°C. Also, 200 µl of the mixture was added to the solution, plus vortex 400 µl of 50% ethanol. The lysed cells were transferred, purified and then centrifuged for 1 min/6000 µg. Washing column was prepared by 500 µL buffer (I&II) and then re-centrifuged at maximum speed about 2 min until ethanol was completely removed. The purified DNA was stored at 20°C. Preparation of the plasmid is accomplished with the DNA plasmid GeneJet mini prep kit (Thermofisher Scientific, USA). The grown culture is placed in a 1.5 ml micro-centrifuge tube and then re-centrifuged at 12,000 xg for 2 min. The pellet is re-suspended in 250 µl of the ice-cold resuspension buffer included in the kit; thereafter, the tube is inverted approximately 56 times and mixed. Further, the tube was incubated for 5 min at 37°C. The supernatant was transferred and centrifuged at 10,000 xg/30 s, followed by rinsing of the pellet with 500 µl buffer (included in the kit) and further centrifugation at 10,000 xg for 30 s. The DNA plasmid was eluted using 50 µl of preheated double distilled H_2O, incubated for 3 min at 37°C, then centrifuged again at maximum rate (14000 xg) for 30 s. For amplification of gene, a PCR reaction was used as follows: 1 µl purified gene material (genomic DNA / plasmid prep), 2.5 µl $MgCl_2$, 5 µl buffer, 1 µl primer (as listed in Table 1), 0.25 µl enzyme mixture of Taq polymerase and 0.5 µl dNTP are combined with free nuclease water and made up to a total volume of 25 µl. Also carried out was lysis of PCR product with 0.5 µg/ml agarose gel and ethidium bromide (1%), as well as size determination of lysate using 100 bp ladder of DNA. Afterwards, the gel was run at 80 V for 50 min and documentation was done by the gel system (Biometra, Göttingen, Germany).

The DNA fragments extraction from agarose gels was as follows: DNA fragments were eluted from agarose gels by DNA Kit (Thermofisher Scientific, USA). Fragmentation is prepared by UV light and stored in a 1.5 ml tube. It was then centrifuged at 13000 xg for 2 min. Further, the column was washed using 700 µl buffer and then re-centrifuged at 1 min at 37°C. 50 µl buffer was added, eluted with a spin column filter, held at 37°C for 1 min, and then centrifuged at 13000 xg for 2 min.

RESULTS

E. coli prevalence

Figure 1 shows that about 100 chicken meat samples were tested on *E. coli*, with 40% found to be positive samples, while the remainder did not show the presence of *E. coli*.

Table 1. Gene amplification primers list.

Target Gene	Primer ID	Primer sequence
Shiga-like toxin (SLT)	SLT F:	5'-AAGAAGATGTTTATGGCGGTTT-3'
	SLT R:	3'-GTCATTATTAAACTGCACTTCAGCA-5'
Heat-labile toxin (LT)	LT F:	5'-ATTGACATCATGTTGCATATAGGTTAG-3'
	LT R:	3'-ACATTTTACTTTATTCATAATTCATCCCG-5'
Ciprofloxacin resistance gene	aac (6')-F:	5'-TTTATTATTTTTAAGCGTGCATAATAAGCC-3'
	aac (6')-R:	3'-TTAAGACCCTTAATTGTTGGGATTT-5'
Gentamicin resistance gene	aac C2-F:	5'-CATACGCGGAAGGCAATAAC-3'
	aac C2-R:	3'-ACCTGAAGGCTCGCAAGA-5'

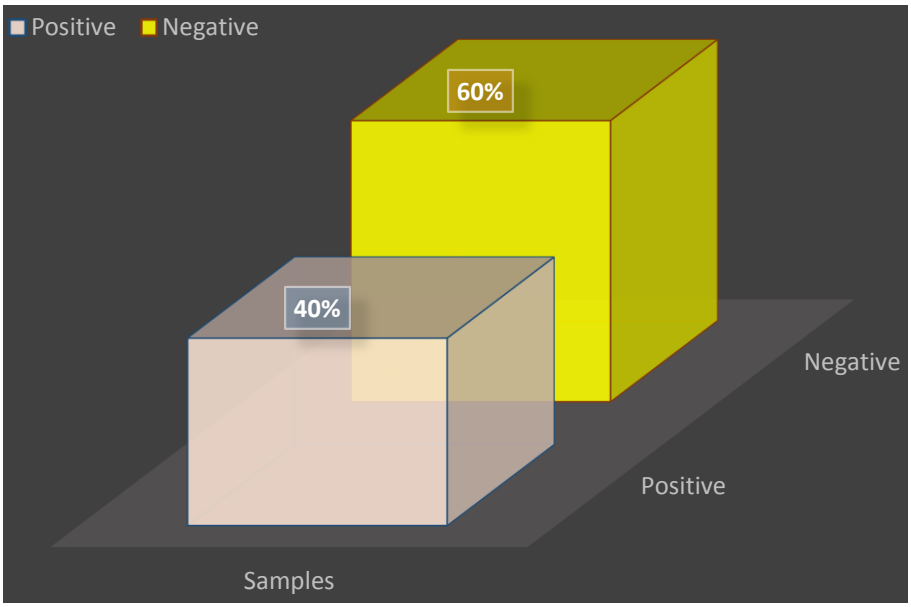

Figure 1. *E. coli* isolate detected incidence.

Antibacterial pattern of *E. coli* resistance:

Investigation of the antimicrobial susceptibility was performed using 12 antibiotics from 6 different antibiotics families, with the test applied against twenty *E. coli* which were isolated from chicken samples. The obtained results were shown in Table 2. The antibiotics selected for *E. coli* were: the most effective antibacterial agents associated with sulfonamides; (20/20) 100% trimethoprim, 80% (16/20) sulfamethoxazole, followed by cephalosporins- about 70% (14/20) cefepime, 65% (13/20) cefotaxime; tetracycline included- 50% (10/20) tetracycline, and 40% (8/20) doxycycline. In the tested antibacterial class, the human family showed weak activity against *E. coli* as follows: Quinolones- (4/20) 20% ciprofloxacin, (4/20) 20% flumekin; aminoglycosides; - 3/20 (15%) gentamicin, (2/20) 10% streptomycin, and β-lactams based on the weakest member- Penicillin (10%) 2/20, and ampicillin (0%).

Serological results of *E. coli* isolates

About 20 *E. coli* were classified serologically in Figure 2. As a result, the 10/20 (50%) isolate was STEC (O157: H7), the 6/20 (30%) isolate was ETEC (O142), and the 2/20 (10%) isolate was (O26: H11) of EHEC, 2/20 (10%) EPEC (O55: H7). The screening for SLT is described in Figure 3. Subunit B of SLT gene showed the uniform genomic DNA band with about 300 bp. of molecular weight. The results show that strain 1 had minimal amplification compared to strain 2. Fragments of

Table 2. Antibacterial *E. coli* susceptibility from chicken meat isolates.

Antimicrobial family	Antibacterial agents	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Sulfonamides	Trimethoprim	20	100	0	00	0	00
	Sulfamethoxazole	16	80	2	10	2	10
Cephalosporins	Cefepime	14	70	4	20	2	10
	Cefotaxime	13	65	4	20	3	15
Tetracycline	Tetracycline	10	50	0	00	10	50
	Doxycycline	8	40	2	10	10	50
Quinolones	Ciprofloxacin	4	20	4	20	12	60
	Flumequine	4	20	6	30	10	50
Aminoglycosides	Gentamicin	3	15	5	25	12	60
	Streptomycin	2	10	5	25	13	65
β-lactame	Penicillin	2	10	6	30	12	60
	Ampicillin	0	00	7	35	13	65

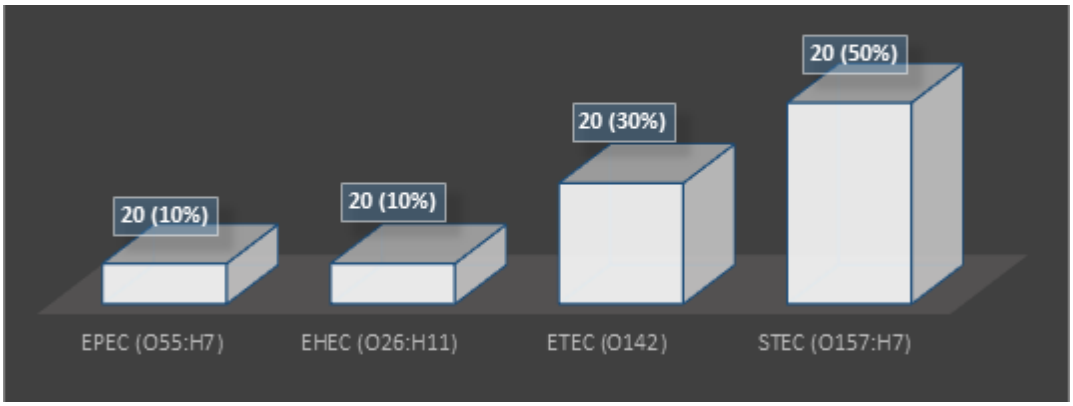

Figure 2. Prevalence of detected *E. coli* serotypes. EPEC: Enteropathogenic *E. coli;* EHEC: Enterohemorrhagic *E. coli;* ETEC Enterotoxigenic *E. coli;* STEC: Shiga Toxin *E. coli*.

approximately 200 bp were estimated in (1) and (2) strains. Figure 4A was confirmed as heat-labile toxin (LT) gene while (B) represented plasmid. The molecular weight of the target gene was about ~ 200 bp of strains (1) and (2). Gentamicin screening resistance was revealed in Figure 5 where the gentamicin gene resistance (aac C2) segment was found in the strain as a segment with a molecular weight of approximately 856 bp; however, a molecular weight of approximately 300 bp was detected. The screening for ciprofloxacin resistance is shown in Figure 6. Low Ciprofloxacin resistance genes were investigated in plasmid and genomic of tested strains. The 1 kb band was detected obviously in strain 1, but not in strain 2. In the case of the plasmid preparation, the gene target amplification was not detected.

DISCUSSION

Bacteriological investigation of 100 chicken meat samples agreed with Partridge et al. (2018) who reported 35.5% from examined Mexican chicken; Wu et al. (2018), which isolated 35.0% *E. coli* from chicken; Ngullie et al. (2011) who reported 31% in Indian chicken examination and Sato et al. (2010) who recorded about 20% for US chicken meat. Also, Shaltout et al. (2020) reported 13.33% *E. coli* from Egyptian chicken; lesser percentages of *E. coli (*11.1%) were reported by Tomova et al. (2018) in Nigerian chickens; Liu et al. (2016) recorded 10.60% from Croatian chicken meat; while Jakabi et. al. (2002) recorded 9% *E. coli* from Chinese chicken. Further, Deng et al. (2016) and Schulz et al. (2015) isolated about

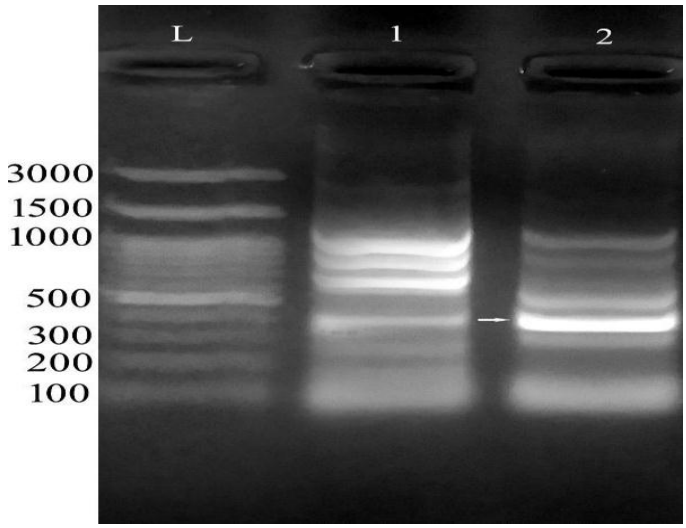

Figure 3. Screening of the Shiga-like toxin (SLT) (B) subunit. Strain 1 has low amplified genes density. Lane (L) showed a DNA ladder standard between 100-3000 bp.

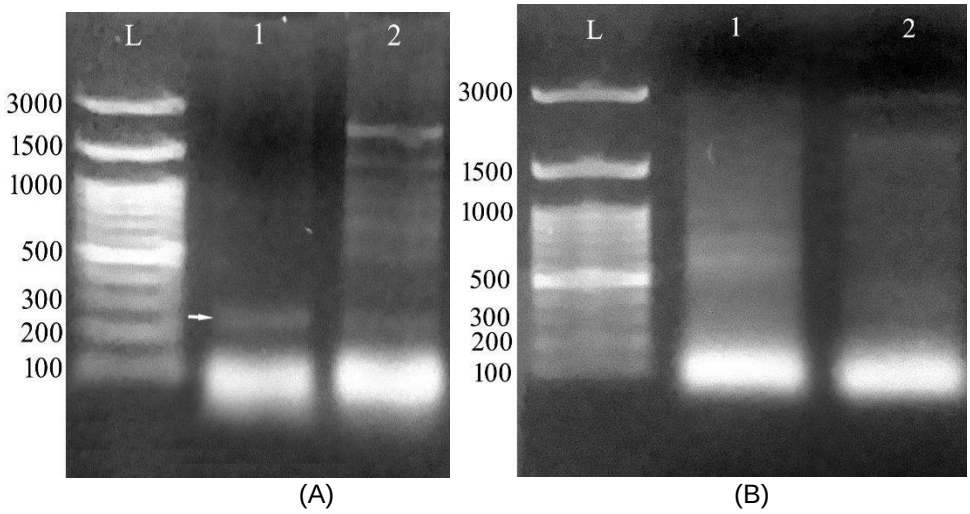

Figure 4. (A) Declared heat-labile toxin (LT) gene, (B) represented plasmid. The molecular weight of the target gene is about ~ 200 bp of strains (1) and (2).

5.92% of Saudi Arabian chicken meat. Lowest percentage was recorded by Collins (2000) who reported about 1.56% from Moroccan chicken. This indicates suboptimal hygiene practices at the various stages such as slaughter, handling practices, transporting, and during meat processing; leading to the presence of this microbe in processed chicken meat-and bone meal (Schulz et al., 2015). *E. coli* is also found in animals and human gastro-intestinal tract. Detection of this pathogen in well prepared chicken foods indicates fecal contamination, which on the other hand, indicates the possible presence of other harmful organisms such as bacterial (*Salmonella, Shigella, Campylobacter*) (Collins, 2000).

Antibacterial drugs are used in prevention and/or treatment, in addition to their use as chickens' growth promoters. The benefits were achieved when the antibacterial agent were properly selected. Antibacterial susceptibility testing against different *E. coli* (n = 20) collected from chicken meat samples showed the patent antibiotics associated with sulfonamides were 100% trimethoprim (20/20), sulfamethoxazole 80% (16/20), (14/20) 70% (cephalosporins and cefepime), (13/20) 65% cefotaxime; while in the case of tetracycline usage the results showed the following: (10/20) 50% tetracycline,

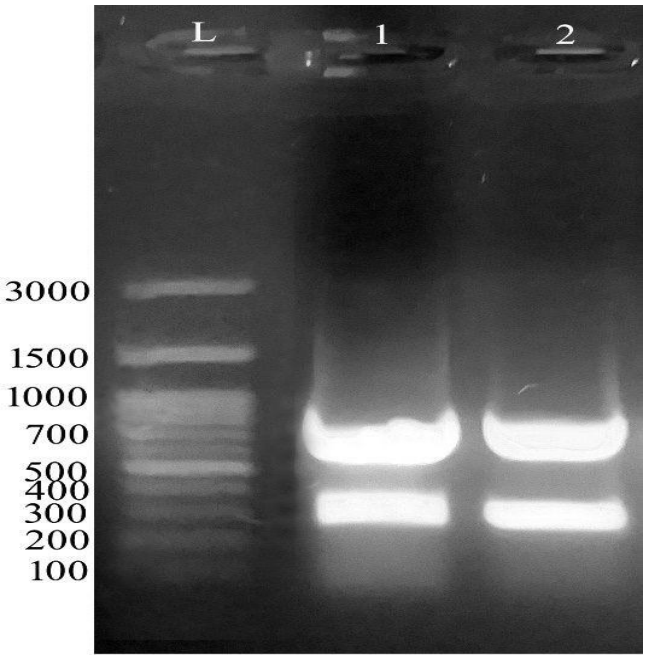

Figure 5. Screening of Gentamicin-resistance gene: declared ~ 856 bp molecular weight of the Gentamicin-resistance (*aac* C2) gene fragment. A minor band was declared at 300 bp in strains 1 and 2.

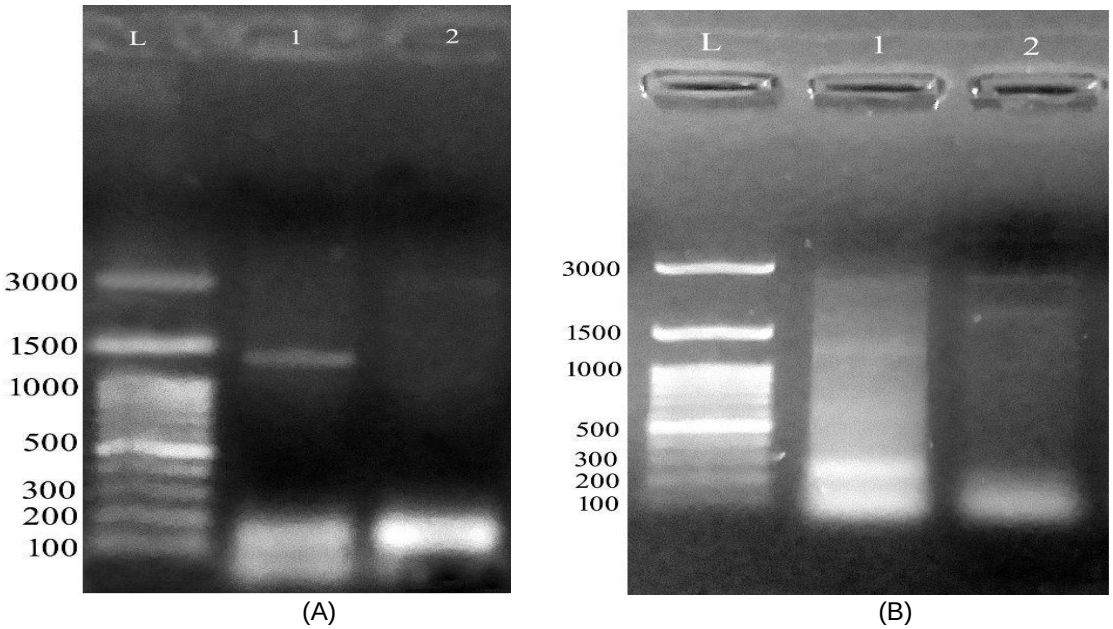

Figure 6. 1 kb size declared the Ciprofloxacin-resistance screening gene where (A) represented the genomic of strain (1) and (B) documented the plasmid amplification.

and (2/20) 10% streptomycin. The weakest member based on β-lactam family was as follows: (2/20) 10% penicillin and (0%) ampicillin. The obtained results agreed with CDC (2019). Nearly same results was observed by Younis et al. (2017) who reported 100% of resistance against penicillin, 95.8% against cefepime and 94.5% amoxicillin against *E. coli*. Ammar et al. (2015) described *E. coli* antibiotics resistance as caused by the

presence of plasmid genes. Adeyanju and Ishola (2014) and Bie et al. (2018) stated about 90% *E. coli* resistant to ampicillin, tetracycline, cephalexin, trimethoprim, sulfametozazole, streptomycin, and gentamicin.

Ramadan et al. (2016) showed multidrug resistance to aminoglycosides, tetracyclines, sulfonamides and β-lactams against *E. coli*. Eid and Erfan (2013) and Mohamed et al. (2014) almost informed the *E. coli* resistance against β-lactams while Li et al. (2020) found that *E. coli* was highly resistant against sulfadiazine, gentamicin, amoxicillin, sulfadiazine, ampicillin, tetracycline, ceftriaxone and chloramphenicol. Zhang et al. (2012) documented that about 60% *E. coli* which were isolated had resistance against fluoroquinolones whereas Tang et al. (2011) found that about 35.0, 36.8, and 34.1% of ciprofloxacin, norfloxacin, and enrofloxacin were resistant.

Serological test of 20 *E. coli* isolates recorded about; STEC (O157: H7)10/20 (50%), ETEC (O142) 6/20 (30%), EHEC (O26: H11) 2/20 (10%) and EPEC (O55: H7) 2/20 (10%). SLT was as follows: B subunit (SLT) gene showed uniform segment in DNA genome at 300 bp molecular weight.

The results showed in strain (1) had the less amplification detection compared to heat labile toxin (LT) screening. Fragments of approximately 200 bp were recorded in both (1) and (2) strains. The resistant gentamicin gene was (*aac C2)* fragment documented with a molecular weight 856 bp while the small band has 300 bp molecular weight. The resistant Ciprofloxacin genes were screened on both plasmid and genomic preparations of the tested strains. Another band was detected at about 1 kb in strain (1), but not in strain (2). In the case of the plasmid preparation, target gene was not found in the isolated strain. According to Momtaz and Jamshidi (2013), O serotypes, especially O2, O1, O8, O18, O15, O35, O88, O115, O78 and O109 were the most detected serotypes. Ying et al. (2020) isolated enteropathogenic and *eaeA* of *E. coli* gene which were nearly identical to *eae* genes of O157: H7; O55: H7 and EHEC. Kakoullis et al. (2019) detected SLT gene was giving false negative results. On the other hand, HECO157 was detected by the 60MDa plasmid. Lagerqvist et al. (2020) detected (SLT I, II and *eaeA)* genes indicating the occurrence to the EHEC (O157) strain. Yang et al. (2020) documented the *Stx* gene detected in EHEC strains. The virulence genes include the extra-intestinal infectious genes: (*afaD8, Cdt2, cdt3, traT, eisen, bmaE, iutA, iucD).* Villegas et al. (2013) detected *etpD* gene in ETEC strain. Further, the intestinal hemorrhage was caused by EDL933 and RIMD 0509952 while *fmH* gene was considered as a non-virulent gene in different *E. coli* strains (Momtaz and Jamshidi, 2013).

Conclusion

The study demonstrated the presence of *E. coli* pathogenic genes from chicken meat samples, including various somatic capsules and antigenic genes. Managing STEC is too essential as it poses hazards to chicken consumers. *E. coli* presence, mainly, in our daily meals is a public health concern and food biosafety issue. It is advisable to ensure proper hygiene measures when slaughtering, handling and/or processing chicken carcasses. It is therefore recommended that unnecessary use of antibacterial drugs in living chickens and humans be avoided to forestall the emergence of new antibacterial resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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