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Full Length Research Paper

Studies of *Salvia officinalis* leaf extract on some biochemical parameters in rats induced with overdosed-tramadol

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The aim of the study was to evaluate the effect of *Salvia officinalis* leaf extract in overdosed-tramadol induced rats. Standard methods of analysis were used for the study. Fifty rats were divided into Group A (Control) - feed and water only, Group B-induced with tramadol at 50 mg/kg body weight, Group C-induced with tramadol + 150 mg/kg of *S. officinalis* leaf extract, Group D-tramadol + 300 mg/kg *S. officinalis* leaf extract, and Group E-tramadol + 400 mg/kg *S. officinalis* leaf extract. The results showed high concentrations of phytochemical and trace elements at various concentrations. There were increases in aspartate amino transaminase, alanine aminotransferase, gamma gluamyltransferase, alkaline phosphatase and in total protein, albumin, total bilirubin, conjugated bilirubin, sodium, potassium, chloride, bicarbonate, urea, calcium, creatinine, total cholesterol, magnesium, hemoglobin, packed cell volume, and total white blood cell count at a significant difference ($p < 0.05$) in Group B when compared with Group A. However, in Groups C, D and E, there was statistically significant decrease ($p < 0.05$) in aspartate amino transaminase, alanine aminotransferase, gamma gluamyltransferase, alkaline phosphatase and total protein, albumin, total bilirubin, conjugated bilirubin, sodium, potassium, chloride, bicarbonate, urea, calcium, creatinine, total cholesterol and magnesium compared with the Group B. The study showed that *S. officinalis* leaf could be of an unalloyed health benefits in the management of tramadol-induced toxicity in rats.

Key words: Tramadol, *Sage*, phytochemicals/elements, markers, rats.

INTRODUCTION

Tramadol (TD) is a synthetic opioid analgesic agent, used parenterally and orally for the treatment of moderate to severe pain in humans (Miotto et al., 2017; Mohamed and Mahmoud, 2019). Tramadol is an ideal analgesic during and after day case surgery and in patients with acute ureteric spasm (Elkhateeb et al., 2015). Tramadol is responsible for life-threatening poisonings, resulting in consciousness impairment, seizures, agitation and respiratory depression (Hassanian-Moghaddam et al., 2013; Owoade et al., 2019). Scientists recommended that tramadol toxic effects should be kept in mind during long term therapy especially in large doses (DePries et al.,

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2015). There are about 35 million users of opioids globally and such opioids like tramadol are known to cause the greatest negative health impart (UNODC, 2017). Previous study observed that overdosed tramadol in man induces hypokalaemia, diarrhea, certain nervous disorder and maintenance of neuro-muscular irritability (Carroll et al., 2006).

Common sage (*Salvia officinalis*) plant, a perennial, evergreen subshrub, native to the Mediterranean region is one of the most commercially important species within the Lamiaceae family (Avato et al., 2005; Ghorbani and Esmaeilizadeh, 2017; Devin et al., 2021). It is found and cultivated world over (Raal et al., 2007; Ghorbani and Esmaeilizadeh, 2017). And it thrives well in Nigeria particularly within the semi urban areas of Vom-Jos, Plateau State (Figure 1). It has become a target for the search of the biologically active compounds and new drugs as it shows a broad range of medical activities (Padhye et al., 2008; Bommer et al., 2011; Keshavarz et al., 2011; Khalil and Li, 2011; Khan et al., 2011; Hamidpour et al., 2014). *S. officinalis* has enjoyed a reputation in Asia and Latin America for traditional medicine for treating all kinds of ailments (Garsia et al., 2016). Presently, the essential oils of it is known to have biological and antioxidant properties (Stagos et al., 2012; Kontogianni et al., 2013; Garsia et al., 2016; Alimpi et al., 2017). It also has anti-inflammatory and cytogenetic effects (Al-Barazanjy et al., 2012; Rodrigues et al., 2012; Alimpic et al., 2015). As tramadol is metabolized mainly in the liver, some researchers (Watson et al., 2004; Jensen-Ortho et al., 2005; Raffa et al., 2008) stated that its long-term therapy particularly in overdose is associated with hepatotoxicity with its corresponding kidneys toxicity especially as tramadol and its metabolites are released via the kidneys.

MATERIALS AND METHODS

Collection of plant and preparation of extract

Fresh samples of sage (*S. officinalis*) were obtained from farmlands in Vom-Jos, Plateau State, Nigeria. The plant was identified, authenticated, and a voucher number (20/21/00121) was given it and stored for future reference (Figure 1). The leaves were removed from the stalk and air dried in the laboratory for 21 days. The dried plant-leaves samples were well ground and sieved into a fine powder from which an extract was made for the experiment. 30 g of the ground leaves was kept in an air-tight container for phytochemical and analytical studies while another 50 g was weighed and added to 400 ml of absolute methanol for 3 days. The mixture was then filtered using muslin cloth and later concentrated using Soxhlet apparatus. Then the extract obtained were kept in airtight sample bottles, labeled and stored in the refrigerator for animal experiments.

Phytochemical analysis

Quantitative phytochemical analysis

Tests for tannins, terpenoids, cardiac glycosides, flavonoids, alkaloids, phenolics, and saponins were carried out using standard methods (Marcono and Hasenaira, 1991).

Test for Tannins

One gram of sample was extracted with 25 ml 80:20 acetone: 10% glacial acetic acid for 4 h. It was then filtered and measured at 500 nm absorbance. The absorbance of the reagent blank was also measured. A standard graph with 10, 20, 30, 40, and 50 mg/100 g of tannic acid was made (Marcono and Hasenaira, 1991).

The concentration of tannins was read taking into consideration the dilution factor.

Test for terpenoids

One gram of sample was weighed into 250 ml beaker and 10 ml petroleum ether was added. It was allowed to extract for 15 min and filtered. The absorbance was then read at 420 nm (Marcono and Hasenaira, 1991).

Test for cardiac glycosides

One gram of sample with 40 ml of water was extracted and placed in an oven at 100°C for 15 min. Then, to 1 ml of the extract dissolved in 5 ml of water was added 2 ml of glacial acetic acid followed by one drop of iron chloride ($FeCl_3$) and 1 ml of H_2SO_4. The absorbance was then measured at 410 nm (Marcono and Hasenaira, 1991).

Tests or flavonoids

One gram of the sample was extracted with 10 ml of 80% methanol and left to stand for 2 h. It was filtered through Whatman filter paper into a Petri-dish, evaporated to dryness in an oven at 40°C and weighed (Marcono and Hasenaira, 1991).

Test for alkaloids

One gram of each sample (W) was extracted with 20 ml of 10% acetic acid in ethanol, mixed and allowed to stand for 4 h. The extract was filtered through Whatman filter paper. The filtrate was evaporated to about a quarter of its original volume and one drop of concentrated ammonia was added. The extract was filtered through weighed (W1) Whatman filter paper.

The filter paper was dried in the oven at 60°C. The dried filter paper was weighed to a constant weight (W_2) (Marcono and Hasenaira, 1991).

$$\% \text{ Alkaloids} = (W_2 - W_1)/W \times 100/1$$

Test for phenolics

Two grams of each sample were extracted with 20 ml of acetone, 0.5% formic acid for 2 min and was filtered. 2 ml of the extract was mixed with 0.5 ml Folin-Ciocalteau reagent, mixed for 15 s and allowed to stand at 40°C for 30min to develop a colour. The absorbance was measured at 765 nm and expressed as mg/g Gallic Acid Equivalent (GAE) (Marcono and Hasenaira, 1991).

Test for Saponnins

One gram of each sample was dispersed in 15 ml of 20%

Figure 1. *S. officinalis* leaves.
Source: Photograph of *Salvia officinalis* leaves taken before drying the leaves

ethanol. The suspension was put inside the water bath at 55°C for 4 h. The mixture was filtered and the residue re-extracted with another 15 ml of 20% ethanol twice. The extract was reduced to about 5 ml in the oven. The concentrate was transferred into a 250 ml separating funnel and 5 ml of petroleum ether was added and mixed vigorously. The petroleum ether layer was discarded and 3 ml of butanol was added to the aqueous layer. The extract was washed twice with 5 ml of 5% sodium chloride. The remaining solution was poured into a weighed Petri-dish, evaporated to dryness in the oven and the residue weighed (Marcono and Hasenaira, 1991).

Analysis of elements

The major trace elements comprising iron, manganese, copper, fluorine, chromium, iodine, selenium, molybdenum, cobalt and zinc were determined according to the method of Shahidi et al. (1999). The ground samples were sieved with a 2 mm rubber sieve and 2 g of each of the samples subjected to dry ash in a well cleaned porcelain crucible at 55°C in a muffle furnace. The resultant ash was dissolved in 5 ml of HNO_3/H_2O_2 (1:1) and heated gently on hot plate until brown fumes disappeared. To the remaining material in the crucible, 5 ml of deionized water was added and heated until a colourless solution was obtained. The mineral solution in each crucible was transferred into a 100 ml volumetric flask by filtration through a Whatman filter paper and the volume made to mark with de-ionized water. This solution was used for elemental analysis by atomic absorption spectrophotometer (AAS) and the concentration of each element was calculated on percentage of dry matter.

Experimental animals

A total of 50 Wistar albino rats of either sex weighing between 150 and 250 g were used for the study. The animals were fed with standard growers' marsh diet and water *ad libitum*, in a standard wire meshed plastic cages for 14 days prior to commencement of the experiment. The animals were handled according to the guidelines of Institutional Animal Ethics Committee (IAEC) (Rowlands, 2013). The protocol was approved by Faculty of Science Research Ethics Committee, Delta State University.

Tramadol-induced toxicity

Wistar albino rats were divided into five groups comprising ten animals in each group: Group A: control was fed with feed and water daily, Group B: Tramadol (50 mg/kg bwt) with feed and water, Group C: Tramadol + 150 mg/kg of sage plant with feed and water, Group D: Tramadol + 300 mg/kg of sage plant with feed and water, Group E: Tramadol + 450 mg/kg of sage plant with feed and water.

Tramadol hydrochloride, bought from a registered pharmaceutical store, made into saline solution was given to the animal through oral route using oral gauge for 14 days after acclimatization (Rowlands, 2013).

Sample collection

At the end of the treatment period the animals were sacrificed 48 h following the last given dose. And at the time of sacrifice, their weights were taken and then cervical decapitation was conducted before laparotomy section was carried out. Blood samples were collected from vena cava and heart into EDTA bottles for full blood count and plane tubes for clotted samples for biochemical analysis. Each plane tube was properly labeled, centrifuged at 4000 rpm for 10 min for serum separated and stored at −8°C pending biochemical analysis using spectrophotometric methods with reagents. The liver, brain, kidney and heart were taken for observations.

Determination of body weight

Body weight of experimental animals was determined at day 0 (before administration) and subsequent days and on the last day of experiment. Dose of Silymarin and extract was given to each rat according to their body weight, respectively (Lipscombe et al., 2021). Percentage weight gain was later calculated as follow:

$$Percentage\ weight\ gain\ (\%) = \frac{Final - Initial\ body\ weight\ (g)}{Initial\ body\ weight\ (g)} \times 100$$

Biochemical assays

The serum of each rat was analyzed for the following biochemical

Table 1. The mean Phytochemicals concentration in mg/100g of *Sage officinalis* leaf extract.

Phytochemical	Tannins (mg/100 g)	Terpenoids (mg/100 g)	Cardiac Glycosides (mg/100 g)	Flavonoids (mg/100 g)	Alkaloids (mg/100 g)	Phenolics (GAE/g)	Saponins (mg/100 g)
Mean value	518.21	321.00	145.00	4288.00	3863.00	60.81	2450.00

Source: Author

Table 2. The mean values of elemental concentration (mg/100g) of *S. officinalis* leaf.

Fe^{++}	Mn^{++}	Cu^{++}	F^{-}	Cl^{-}	I^{-}	Se^{++}	Mo^{++}	Co^{++}	Zn^{++}
9.77	0.05	0.71	0.02	56.45	0.42	0.04	0.04	0.03	0.52

Source: Author

parameters (assays): AST (E.C. 2.6.1.1) and ALT (E.C. 2.6.1.2) were determined by Reitman and Frankel (1957), GGT{E.C. 2.3.2.2} was determined by Gjerde and Marland (1985), ALP (E.C. 31.3.1) by Kind and King (1954), TP and ALB by Reinhold (1953), TB and CB by method of Malloy and Evelyn (1937), UR by March et al. (1965), CR by Reinhold (1953), TCHOL by Robinson & Pugh (1958), sodium level by Buzanovskii (2018), potassium level by Wong et al. (1985), bicarbonate by Sobel and Eichen (1952), chloride by Henry (1964), while HB, PCV and TWBC were determined by the method as documented by Dacie and Lewis (2001).

Statistical analysis

All the data obtained were expressed as mean ± standard error of mean (SEM) and was subjected to ANOVA analysis using Dunnett's *t*-test. A p-value of less than 0.05 was considered significant.

RESULTS

The results in (Table) 1 show the concentrations of phytochemicals in common sage plant leaf with flavonoids having the highest concentration of 4288 mg/100 g, followed by alkaloids 3863 mg/100 g, saponins 2450 mg/100 g, tanins 518.21 mg/100 g, terpenoids 321.00 mg/100 g, cardiac glycosides 145 mg/100 g and phenolics 60.81 GAE/g. (Table) 2 shows the elemental composition of *S. officinalis* leaf with Cl^{-} having the highest concentration of 56.45 mg/g, followed by Fe^{2+} 9.77 mg/g, Cu^{2+} 0.71 mg/g, Zn^{2+} 0.52 mg/g, I^{-} 0.42 mg/g, Mn^{2+} 0.05 mg/g, Se 0.04 mg/g, Co^{2+} 0.03 mg/g, Mo^{2+} 0.04 mg/g and F 0.02 mg/g in that order.

The results in (Table) 3 show the mean concentration of body weights of the experimental rats before and after induction with tramadol and tramadol plus *S. officinalis* leaf extract at various concentrations. The group A-103.63 ± 16.25^{b} shows significant increase in the body weight ($p< 0.05$) when compared with group B (Tramadol) induced rats-97.93 ± 4.65^{b}. The group A control (103.63 ± 16.25^{b}) also shows significant decrease ($p< 0.05$) across the Groups C (115.43 ± 12.47^{b}) and Group D (117.43 ± 13.28^{b}) rats while the Group A (103.63 ± 16.25^{b}) is not significantly different ($p > 0.05$) when compared with Group E (104.83 ± 17.18^{a}). However, there are statistical differences ($p< 0.05$) in the body weight before and after tramadol induction plus *S. officinalis* extract in addition at different levels in comparison with the group B and this is particularly visible at the tramadol plus 300 ml *S. officinalis* leaf extract addition.

Table 4 shows the effect of *S. officinalis* leaf extract on various biochemical parameters on tramadol induced rats. From the table, there are statistical significant increases in activity/increase ($p < 0.05$) in the liver function tests parameters: AST, ALT, Alk. Phis, GGT, TP, ALB, TB, and CB in group B compared with normal control group. There was also significant increase ($p < 0.05$) in Serum Electrolytes levels: Na^{2+}, k^{2+}, Cl, HCO_3^{-} in group B compared with the group A. Equally, statistical comparison between group A and group B with regards to the kidney function tests: Serum UR, Serum CR as well as Ca^{2+}, UA, TChol biochemical parameters levels revealed significant difference ($p < 0.05$). Also, the mean concentration of TWBC in rats induced with Tramadol showed significant difference ($p < 0.05$) compared with the group A rats. While the level of the biochemical parameters: HB and PCV in the group B showed significant decrease ($p < 0.05$) in comparison with the group A. Equally, there is no significant difference ($p > 0.05$) in the level of Mg^{2+} in the group A compared with group B. However, significant statistical reduction ($p < 0.05$) exist in the various biochemical parameters except in the levels of HB and PVC where there are significant increase ($p < 0.05$) analyzed in the groups with tramadol plus *S. officinalis* leaf extract at different levels of administration compared with the group B that was induced with tramadol alone.

DISCUSSION

Enormous interest abounds in the use of herbal remedies

Table 3. Effect of Sage plant leaf on body weight of Rats before and after induction of Tramadol.

Biochemical parameter	Group A Normal Feed & Water	Group B Tramadol	Group C Tramadol +150 mg Sage L	Group D Tramadol +300 mg Sage L	Group E Tramadol +450 mg Sage L
Before	94.00±5.45[a]	91.33±6.11[a]	95.67±20.50[a]	93.00±16.64[a]	93.33±4.16[a]
After	103.63±16.25[a]	97.93±4.65[b]	115.43±12.47[c]	117.43±13.28[c]	104.83±17.18[a]

Values are expressed in Mean ± S.E.M., n=10. Values sharing the same superscript in the row did not differ significantly ($p > 0.05$). Values sharing different superscript in the same column are statistically significant ($p < 0.05$).
Source: Author

Table 4. Effect of Sage plant leaf on the various biochemical parameters on Tramadol induced rats.

Biochemical parameter	Control Group A	Group B Tramadol induced	Group C Tramadol +150 mg Sage L	Group D Tramadol +300 mg Sage L	Group E Tramadol +450 mg Sage L
AST	12.50±2.28[a]	23.60±2.90[b]	13.15±2.18[a]	14.98±3.18[c]	15.67±2.19[c]
ALT	10.35±2.28[a]	18.56±3.10[b]	11.00±2.16[a]	13.10±2.16[c]	13.87±3.12[c]
GGT	29.01±2.18[a]	41.14±3.19[b]	31.28±2.19[a]	30.17±3.12[c]	30.10±3.18[c]
ALP	15.0±3.08[b]	48.0±5.34[a]	17.3±3.89[b]	22.3±1.10[c]	23.67±3.63[c]
TP	6.00±0.53[a]	4.33±0.12[b]	5.90±0.30[a]	7.57±0.40[c]	7.53±0.40[c]
ALB	3.37±0.39[a]	3.93±0.29[c]	3.23±0.11[b]	3.23±0.04[b]	3.2±0.14[b]
TB	0.28±0.01[b]	2.83±1.56[a]	0.26±0.01[b]	0.21±0.3[b]	0.20±0.05[b]
CB	0.52±0.29[b]	2.4±0.14[a]	0.76±0.01[b]	0.43±0.19[b]	0.29±0.00[b]
Na^{2+}	135.00±3.51[a]	145.00±0.500[b]	131.67±2.89[a]	140.67±1.15[a]	146.67±7.64[a]
K^{2+}	3.30±0.61[a]	3.70±0.40[a]	3.77±3.38[a]	3.93±±0.53[a]	3.63±0.25[a]
Cl^{-}	105.00±2.18[a]	132.45±3.12[b]	108.06±2.18[a]	120.95±3.14[c]	119.81±3.21[c]
HCO_3	26.67±3.51[a]	26.00±3.00[a]	25.67±1.15[a]	26.00±1.00[a]	25.00±3.61[a]
UR	30.00±10.00[a]	58.57±3.21[b]	25.43±2.18[c]	27.70±1.18[c]	29.85±3.71[a]
Ca^{2+}	7.67±0.40[a]	9.27±1.66[b]	8.27±1.69[b]	7.73±0.32[a]	10.30±1.17[c]
UA	7.00±0.00	12.70±2.61b	4.43±0.86a	4.90±1.10a	4.90±2.78a
TChol	4.44±0.38[a]	8.34±0.36[b]	4.87±2.14[a]	5.90±3.18[c]	5.91±3.18[c]
Mg^{2+}	1.88±0.06[a]	1.95±0.14[a]	1.85±0.40[a]	2.04±0.10[b]	2.01±0.08[b]
HB	13.86±0.89[a]	8.34±0.36[b]	14.47±0.19[a]	14.14±0.18[a]	13.90±0.38[a]
PCV	0.46±0.01[a]	0.31±0.01[b]	0.47±0.19[a]	0.45±0.01[a]	0.45±0.18[a]
TWBC	8.52±0.41[a]	16.62±0.75[b]	8.98±0.58[c]	8.75±0.61[b]	8.65±0.31[b]

AST = Aspartate amino transaminase, ALT = Alanine aminotransferase, GGT= Gamma gluamyltransferase, ALP = Alkaline phosphatase, TP = Total protein, ALB = Albumin, TB = Total bilirubin, CB = Conjugated bilirubin, Na^{2+} = Sodium, K^{2+} = Potassium, CL^{-} = Chloride, HCO_3 =Bicarbonate, UR = Urea, Ca^{2+}= Calcium, CR =Creatinine, TCHOL = Total cholesterol, Mg^{2+}= Magnesium, HB = Hemoglobin, PCV = Packed cell volume, TWBC = Total white blood cell count.
Source: Author

for the treatment of various diseases in humans worldwide. In this study, the phytochemicals found are similar to the observation of Khiya et al. (2019) who detected polyphenols, gallic tannins, flavonoids, saponins and terpenoids at various levels in the leaves of Moroccan *S. officinalis.* Also, the finding of phenols in the present work is in agreement with the work of Abdulkader et al. (2014) and that of Sarhan et al. (2013) who both documented that *S. officinalis* is rich in polyphenolic compounds. Our findings are in line with the work and observation of El-Feky and Aboulthana (2016) that the biologically active phenolic constituent characterized by aromatic ring with hydroxyl group, is responsible for the various medicinal properties of *S. officinalis*. Equally, the findings are similar to the observation of Khiya et al. (2019) and Kadhim et al. (2016) that *S. officinalis* leaf contains phytochemicals at various levels which confer antioxidant activity to it. From our findings, the mean concentrations of phytochemicals in mg/100 g obtained are far higher than the values reported in *Vernonia amygdaline* leaf (Mokogwu et al., 2014). Also, the mean concentration of the elements in mg/100 g obtained are higher than the values reported in *Vernonia amygdalina* leaf extract (Mokogwu et al., 2014) though from an entirely different species and family. Equally, the detection of phytochemicals: flavonoids, alkaloids, saponins,

phynolic acids, etc., is in line with other works (Dogan, 2004; Slamenova, 2004; Lima et al., 2005; Baranauskiene et al., 2011) that reported that the leaves, roots and water soluble extractions of *S. offisinalis* contain volatile fatty acids, saponins, diterpenes, flavonoids, ohenolic acids, salviatamins, resin and oestrogenic substances.

Trace elements are inorganic substances that act as co-factors and are required in the body in minute amount (Darwish, 2014). The values of trace elements found in our work are similar to the observations of Darwish (2014) and Abu-Darwish et al. (2010) who noted high contents of trace elements: Cu^{2+}, $^{Fe2+}$, Mn^{2+}, and Zn^{2+} at various concentrations in different areas of Jordan. The findings of the current study are also in agreement with earlier work that reported tangible quantities of trace elements in ginger and *Sage* plants in Algeria (Lamari et al., 2011). The level of Zn^{2+} observed by us is equally in line with that of Stef et al. (2010) who noted very high level of Zn^{2+} in Romania.

In this study, the increase in the body weight of rats after tramadol induction in the presence of *S. officinalis* leaf extract does not agree with the work of Ninomiya et al. (2004) that reported reduction of body weight and accumulation of epididymal fat weight in high-fat diet fed mice after 14 days.

Functions of the liver and the kidneys are impaired in overdosed tramadol metabolism and excretion (Matthiesen et al., 1998). In this study, there is toxicity of the liver in the group B which is a reflection as shown in the increased activity of the aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP) and increase in the levels of total proteins (TP), albumin (ALB), total bilirubin (TB), and conjugated bilirubin (CB). This is similar to the results obtained by other workers (Wu et al., 2002; Atici et al., 2005; Jensen-Ortho, 2005) who reported statistical increase in the activities of AST, ALT and lactose dehydrogenase (LDH) in rats after induction with tramadol. Some researchers stated that AST though a mitochondrial enzyme, its increased serum activity is not specific of hepatic disorder but rather denote persistent cellular injury with other enzyme like ALT (Vazarova et al, 2002). Other works noted that serum ALT, as a cytoplasmic enzyme is relatively specific of hepatic disorder and connotes early hepatotoxicity (Moss and Henderson, 1999). However, GGT is highly specific of hepatotoxicity. Also, sosme researchers opined that the increase activity of these hepatic enzymes could be secondary events following tramadol-induced lipid peroxidation of hepatocytes with the subsequent increase in the leakage of these biochemical markers from the liver (Nehu and Anand, 2005). Equally, the impairment of the kidney functions in the group B as indicated by increased in serum urea, creatinine and variations in the electrolytes levels compared to the group A levels portrays kidney toxicity. This result is in tandem with the reports of other studies (Atici et al., 2005; El-Gaafarawi, 2006). A study suggested that renal insufficiency associated with tramadol may be due to the decreased glomerular filtration rate or secondary to the increase in reactive oxygen species (Noori and Mahboobe, 2010).

In this study, the fact that the administration of *S. officinalis* leaf extract along with tramadol-induced toxicity in rats led to increased HB and PCV levels, is an indication that *S. officinalis* leaf extract could poses immunogenic properties. In the same vain its reduction in total cholesterol level also shows that the extract might have anti-hypertensive properties.

Conclusion

The study shows that the administration of *S. officinalis* leaf extract at proper concentration during tramadol-induced toxicity would be of good benefit to the subjects. Also, the fairly high concentration of phytochemicals along with the valuable trace elements observed in this study portends *S. officinalis* leaf as an unalloyed health benefits in the management of liver and kidney related disorders in humans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The value-chain of cotton industry in Kenya with focus on product stewardship for timely provision of certified quality Hybrid Bt cotton seeds to farmers

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For there to be sustainable revival of Kenya's Cotton Industry, its cotton-value needs to be analyzed, there is need to identify its weak points and implement measures to ameliorate its key constraints. Various authors have developed and analyzed the value-chain of the Cotton Industry in Kenya. This paper reviews important components of the value-chain focusing on strengthening the supply of safe, adequate and quality seeds to smallholder farmers as first and immediate priority. The government of Kenya has portrayed a strong policy support to increase cotton production in cotton growing counties of Kenya. In December 2019, the government approved the first commercial release of improved biotech cotton. The government also enacted laws, regulations and policies to strengthen the cotton value-chain and instituted institutional reforms such establishment of Kenya National Biosafety Authority, the Fiber Directorate, the Cotton Development Authority and revitalization of Rivatex Textile Industry. These excellent efforts need to be synchronized and harmonized for effective and efficient performance of the cotton value-chain especially to enhance the supply of improved quality cotton seeds for smallholder farmers. Because of the introduction of county governments and devolution of agricultural functions from central to county governments, it is imperative that two arms of government synchronize activities in the Cotton Industry to ensure adequate support for smallholder farmers for key services such as extension services, supply of quality seeds and provision of credit.

Key words: Cotton industry, quality cotton seeds, policies, regulations, institutional reforms.

INTRODUCTION

The importance of Cotton Industry in Kenya is based on several premises. Kenya's Vision 2030 identifies cotton as the key sub-sector with the potential to benefit 8 million people in the drier areas of the country that cover

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87% of the country (Gok 2007; Gitonga et al, 2016). The government set up the Cotton Development Authority (CODA) to coordinate the revitalization of the Cotton Industry (Gitonga et al, 2016). Kenya has 52 textile mills of which 15 are operational and operate at less than 45% capacity. The mills use outdated technology and suffer from low skilled labour and productivity (Eleksie, 2017). Kenya has a strong regional position under AGOA with a share of total SSA textile; its apparel exports to the US increased from 16% in 2004 to 37% in 2014 (Eleksie, 2017). Apparel manufacturing in Kenya is the most attractive investment option for global investors of duty-free access to the USA and to the EU under EPA, has well-developed export channels, infrastructure and linkages with large USA buyers, which can prove beneficial for new investors (Eleksie, 2017). In 2015, Kenya's total apparel exports reached USD 380 million and were expected to grow at 5% reaching $400 million in 2016 (Eleksie, 2017). AGOA gives most sub-Saharan Africa (SSA) firms duty free, quota free access to United States, offering a substantial competitive advantage over other textile-apparel exporting countries and trade agreement is pivotal in the growth of the continent's textile-apparel sector (Eleksie, 2017). Kenya's textile and apparel sector has potential to strengthen the country's progress into middle income status and as a source of gainful employment for its fast growing, young population (Eleksie, 2017). Kenya's annual demand for cotton lint was 111,000 tonnes of seed cotton, while the average annual production of seed cotton was 18,000 tonnes from 2005 to 2010 (FAOSTAT, 2012; CODA, 2008). The textile Industry meets the deficit through lint imports from Tanzania and Uganda (Monroy et al, 2012).

The purpose of this review paper is to project scenarios of sustainable delivery of adequate quality Bt cotton hybrid seeds to small holder farmers in Kenya.

METHODOLOGY

Information outlined in this work was obtained from reviews of literature and various reports on the establishment and development of the cotton, textiles and apparel industry in Kenya as well as experiences in supporting and building of the cotton industry.

Information on Kenyan cotton seed system was collected using zoom-based interviews of key stakeholders in biotechnology, biosafety and seeds systems. Information was also obtained from interviews done with experienced representatives of Africa Seed Trade Association, Bayer East Africa, Kenyan Fiber Directorate and Cotton Research. The information was augmented through visit and interviews of two major cotton growing counties in Kenya-Makuweni and Kirinyaga. Interviews were made with heads of Makuweni Cotton Ginnery and Biotechnology farmers' Association (Sobitex) who grew Bt cotton hybrid. Two smallholder farmers were interviewed in Kirinyaga who grow cotton and are involved in growing first round of Bt cotton hybrid. Based on the reviews of reports, visits, interview results, and focused discussions, the various pathways used to deliver Bt cotton seeds to smallholder farmers in Kenya were identified and new alternative scenarios that can be used for the seeds delivery were proposed.

RESULTS AND DISCUSSION

Production of cotton

In Kenya, cotton is produced in five regions: Western, Nyanza, Central, Eastern and Coast which today represent 23 of the 47 County Governments (Figure 1). Western and Nyanza regions receive 1000 to 1500 mm annual rainfall. The cropping season of cotton is March to October and the regions have a potential to grow irrigated cotton. The Eastern and Central regions receive 600 to 1200 mm rainfall annually; the crop lasts from August to October. The Coast Region gets 800 to 1200 mm rainfall annually; the cropping season is from April to November (Monroy et al, 2012).

Most cotton in Kenya is grown by about 140,000 smallholder farmers; they have average yield of 0.53 tonnes of cotton seed per hectare. The output characterized by poor quality cotton fiber (World Bank, 2005; FAOSTAT, 2012). Kenya has about 384,500 ha potential for growing cotton of which 10% is currently under cotton production (World Bank, 2005; CODA, 2008). If this potential land is used for cotton production, the country can produce 200,000 tonnes of seed cotton. However, between 2005 and 2010 the country produced on average of 18,000 tonnes per year, equivalent to 9% of this potential.

Constraints to cotton production

Nine key constraints to cotton production in Kenya include inadequate availability of quality planting seed, availability of substandard agro-chemicals, lack of updated quality assurance protocol and testing equipment, low yields of cotton seed, low gross margin of cotton compared to other enterprises, risk aversion by the private sector and farmers, lack of adequate enabling policy support for the industry, inadequate irrigation facilities and high input costs (Gitonga et al, 2016; FAOSTAT, 2012). The Government of Kenya on realizing the need and urgency to revitalize the Cotton Industry in Kenya has embarked on programs to alleviate these constraints that include policy reforms, improved quality seeds supply to farmers, modernization of cotton textiles, revitalization of ginners among others. This paper reviews these reforms in Cotton Industry emphasizing the provision of adequate supply of quality seeds to farmers.

Cotton value chain in Kenya

Various authors have developed and analyzed cotton value chain in Kenya (Ikiara and Ndirangu, 2003; World Bank, 2005; World Bank, 2004; Monroy et al, 2012; Fibre Crops Directorate, 2021; Malicha and Njoroge, 2020; Njoroge and Wario, 2021). Figure 2 shows the most explicit value chain developed by the Kenya Fibre Crops

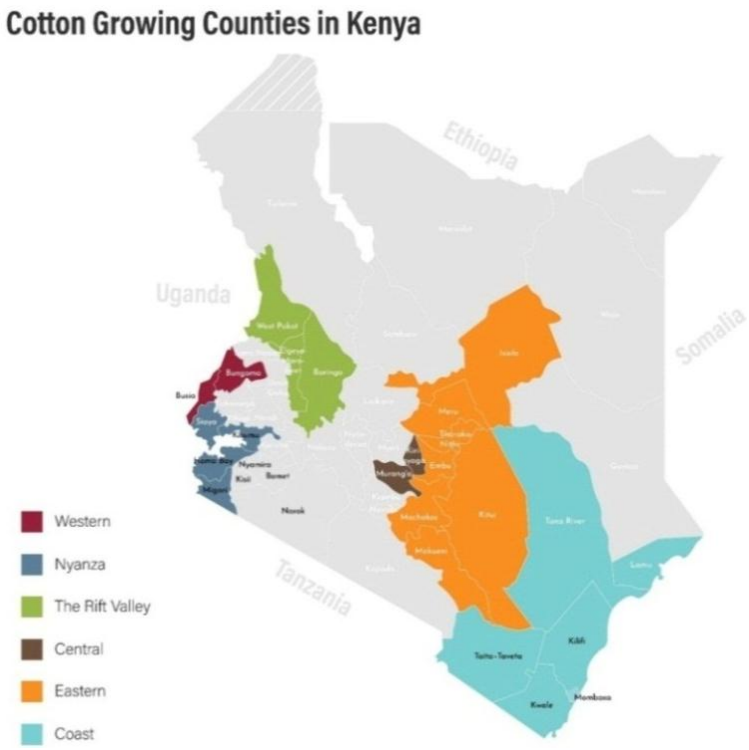

Figure 1. Cotton is grown in small-holder farms in the following regions and Counties. The Rift Valley - Elgeyo Marakwet, Baringo and West Pokot, Eastern - Kitui, Machakos, Makueni, Isiolo, Embu, Meru, Tharaka Nithi; Central - Kirinyaga, Muran'ga, Coast - Tana River, TaitaTaveta, Kwale, Kilifi, Lamu, Nyanza - Siaya, Kisumu, Migori, Homabay; Western - Busia, Bungoma.
Source: Published articles on regions growing cotton in Kenya (Monroy et al, 2012).

Directorate 2021. This later value chain is quite detailedand is categorized into six important components: specific inputs, production, processing, trade and quality control and consumption (Fibre Crops Directorate (2021). The preceding value chain is quite comparable to the World Bank (2005) value chain with five components: cotton farmers, gin operators, textile producers, garment manufacturing and exporters. This paper reviews the components of this value chain in respect to the current interventions supported by the Government of Kenya to revive the Cotton Industry categories; they are policy reforms, institutional reforms, global opportunities, seed system based on emerging technologies of hybrid insect resistant cotton and stacking of other traits such as herbicide and insect resistance.

Policy reforms

The Government of Kenya has formulated several policies, laws and regulations and revised old legislation to spur the development and sustainability of the cotton 2030, The Big Four Agenda (GOK, 2017), The Co-operative Societies Act (Cap 490), The Kenya National industry. These include but not limited to Kenya's Vision Biosafety Act 2009 and Implementing Regulations, The Cotton Act, Cap 335 in 1990 among others outlined subsequently (Gok, 2007; KNBA 2021a, KNBA 20021b).

Kenya Vision 2030 and the Big Four Agenda and Pillar 4-Manufacturing

Kenya Vision 2030 is the country's new development blueprint covering the period 2008 to 2030. It aims to make Kenya a newly industrialized, "Middle income country providing high quality life for all its citizens by 2030" (Gok, 2007). "The Big Four Pillars" is an economic blueprint developed by the government to foster economic development and provide a solution to the various socio-economic problems facing Kenyans (Gok, 2017; Mutuku, 2019). The agenda entails four pillars: food security and nutrition, affordable universal health, affordable housing, and enhancing manufacturing (GoK, 2007). Under Pillar 4, "Manufacturing" the government plans to develop local manufacturing industries that include textiles, leather and

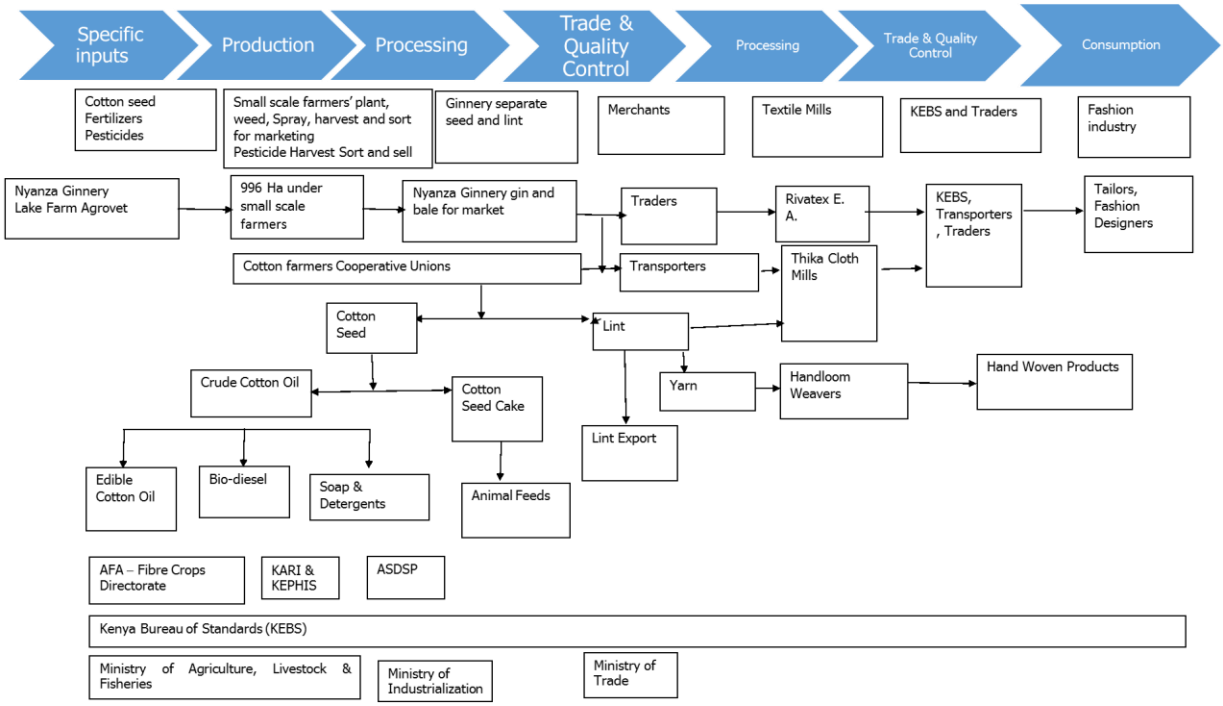

Figure 2. The cotton value chain in Kenya. Published with permission from the Fiber Crops Directorate.
Source: Fibre Crops Directorate-(2021), published with permission.

eliminating impediments to manufacturing (Mutuku, 2019; Mwasiagi et al, 2015; GOK, 2017).

Kenyan biosafety act and implementing regulations

The National Biosafety Authority (KNBA) was established by the Biosafety Act No. 2 of 2009 to provide regulatory oversight, supervision and control over the transfer, handling and use of genetically modified organisms (GMOs) (KNBA, 2021a). The Biosafety Act, 2009 was passed into law by the Kenyan parliament in December 2008 and received Presidential accent on 12 February 2009 (KNBA; 2021b) and Implementing Regulations (KNBA; 2021b). The objectives of this Act are: (1) To facilitate responsible research and minimize risks that may be posed by genetically modified organisms; (2) To ensure adequate level of protection in the development. Commercialized biotech crops are usually approved after thorough review of environmental safety such as gene flow; examples are gene flow studies in sorghum in Kenya (Magomere et al., 2015), impact on non-targets among others (Environmental Protection Authority, 1998; EFSA; 2010) and food safety data covering aspects such as allergenicity and toxicity (FAO/WHO, 2001; EFSA, 2009; Codex, 2009; EFSA, 2010; EFSA, 2011; EFSA, 2017; Kenya BCH-CBD, 2016) and also socio-economic consideration in accordance with international standards. KNBA reviewed these categories of safety data before approving the Bt cotton in Kenya (KNBA, 2019; Monsanto, 2000; USDA, 2002; Kenya BCH-CBD, 2016). In addition, KNBA has developed and approved Genome Editing Guidelines making Kenya the second country in Africa after Nigeria to have regulatory policy over this rapidly emerging technology (Obukosia et al., 2020; KNBA, 2022; NBMA, 2019). Till date, KNBA has provided in the country a regulatory oversight over research, development and commercialization of several biotech crops including maize, cassava, cotton, sweet potatoes, sorghum among others to ensure the technology is safe to humans, animals and the environment (KNBA, 2009; AATF, 2021; CIMMYT, 2022; KALRO, 2020; AHBFI, 2007; Obukosia, 2014; KNBA 2019; KNBA, 2021c; KNBA, 2021b, Henley et al., 2010; Ping et al., 2016, Magomere et al, 2015). Recently, KNBA has approved contained research applying genome editing technology to enhance productivity of several crops including potatoes, cassava, bananas, sorghum among others in Africa (KNBA, 2022b).

This policy reform and experience with the KNBA gives the institution divers versatility, regulatory capacity and oversight over GMOs research, biosafety approvals, including emerging technologies and credit to strong policy support from the Kenya government.

Although Kenya NBA approves the GMO crops based on thorough safety considerations, the crops must further Be assessed under the National Performance Trials (NPT), the auspices of Kenya Plant Health Inspectorate

Services (KEPHIS) and later approved for variety registration and commercialization by the National Variety Release Committee.

Role Kenya plant health inspectorate service

In our recent published paper on "Interface between Event Approval and Commercial release of biotech crops" (Olalekan et al., 2021), we noted that in countries with Seeds Acts such as Kenya (Gok, 2012b), for biotech crops to be commercialized for smallholder cultivation, they go through two key stages of regulatory approval. First, is the biosafety approval and second is approval for varieties registration release/commercialization by conducting National Performance Trials (NPT) (KEPHIS, 2021a) under the auspices of KEPHIS and variety release, registration/commercialization by the National Variety Release Committee (NVRC) (KEPHIS, 2021a; Waturu et al., 2019).

With regard to the recently approved insect resistant cotton "Event Mon 15985", KEPHIS provided oversight over the NPTs of "essentially derived" varieties before they were released in Kenya (KEPHIS, 2021a). In addition, KEPHIS routinely provides Seed Certification services for conventional seeds and is now getting into biotech crops to ensure sustainable supply of quality certified seeds to farmers (KEPHIS; 2021b; KEPHIS, 2921c). Today KEPHIS is providing oversight over NPTs of another biotech crop, Virus Resistant Cassava for Africa (VIRCA) in Kenya. KEPHIS has developed a Guideline for the NPT of Biotech crops and it has adequate institutional and human capacity for regulatory oversight of quality seeds of biotech crops down the value chain (KEPHIS (2021a; KEPHIS, 2021b; Waturu et al, 2019).

Policies and laws and institutions regulating the cotton industry in Kenya

The Cotton Lint and Seed Marketing Act of 1955 was the first legal instrument to regulate the Cotton Industry in Kenya. The Act stipulated the formation of the Cotton Lint and Seed Marketing Board whose main function was to purchase both lint and seed from the ginners and advise the Minister of Agriculture on the prices to be paid by ginners to growers. The Act was repealed by the Cotton Act, Cap 335 in 1990 and established the Cotton Board of Kenya as a State Corporation under the Ministry of Agriculture. The Cotton Act was amended in 2006 to create Cotton Development Authority (CODA) under section 3 (Fibre Crops Directorate, 2021; CODA, 2021; Ruotsi (1989). The Cotton Development Authority is a regulatory state corporation under the Ministry of Agriculture established under section 4 of the Cotton (Amendment) Act 2006. The role of the Authority is to promote, coordinate, monitor, regulate and direct the cotton industry in Kenya (CODA, 2021).

The Cooperatives Policy Framework-Acts, Regulations and By-Laws

Kenya has strong Cooperative Society movement whose role is to ensure the welfare of their members. Three co-operative laws applicable to the co-operatives in Kenya are: The Co-operative Societies Act (Cap 490 of the laws of Kenya) Gok, 2012a), The Co-operative Societies Rules 2008, The Sacco Society Act 2008; The Registered By-laws of the Cooperative Society (GoK, 2013). To operationalize these laws Kenya has "The Sacco Societies Regulations and provision for the Registered By-Laws of the Cooperative Society. The Co-operative Societies Act (Cap 490 of the laws of Kenya) is an approved Act of Parliament governing the constitution, registration, and regulation of co-operative societies. It is the supreme law relating to the operations of the co-operative societies. The current and future Cotton Farmers Cooperative Societies need to be governed by these laws and regulations for proper governance to strengthen the bargaining power of both small scale and forthcoming large scale farmers. Their adherence to this governance and accountability structure will help the cotton farmers' cooperative to eschew previous challenges that may have cost loss of farmers' confidence in their management and cotton industry.

Institutional reforms

The Government of Kenya conducted several institutional reforms aimed to revitalize the Cotton industry in the country: establishment of the Cotton Development Authority (CODA), reconstitution of the Fibre Development Authority, revitalization of Cotton Ginneries and Cotton Cooperative Societies, modernization of cotton textiles notably the Rift Valley Textiles (Rivatex) outlined subsequently (Fibre Crops Directorate, 2021; Abuyeka, 2021; CODA, 2010). In addition, in 2010, Kenya adopted a "New Constitution" that created county governments with delineated functions from the Central National Government abolishing the provincial administrative system (Githinji, 2021).

Role of county and central government in cotton industry in Kenya

In 2010, Kenya adopted a new constitution designated "The Constitution of Kenya 2010 (Githinji, 2021) under which the country adopted a devolved government systems consisting of the National and 47 County Governments (Gok, 2010a) (Figure 1). The Constitution

Article 6(1) divides the territory of Kenya into the forty-seven counties specified in the First Schedule. The governments at the national and county levels are distinct and inter-dependent but conduct their mutual relations based on consultation and cooperation (Article 6:2). The functions of County Governments in Kenya are provided for in Article 186 and assigned in The Fourth Schedule of The Constitution (Githinji, 2021). The function of the county government includes agriculture consisting of (a) crop and animal husbandry, (b) livestock sale yards, (c) county abattoirs, (d) plant and animal disease control, and (e) fisheries (Githinji, 2021). Trade development and regulation, including (a) markets, (b) trade licences (excluding regulation of professions), (c) fair trading practices, (d) local tourism, and (e) cooperative societies. The function of the National Government on the other hand comprises monetary policy, currency, banking (including central banking), the incorporation and regulation of banking, insurance and financial corporations, universities, tertiary educational institutions and other institutions of research and higher learning, agricultural policy, and veterinary policy (Gok, 2010b; KLRC, 2022).

It is evident that this delineation of functions between the national and county governments will affect the ability to create a sustainable cotton industry in Kenya. For example, farmer cooperative, agricultural extension serves which are vital for enhancement of small holder cotton production should be provided by counties. While, improved cotton seed support by National Agricultural Research Stations (NARS) or universities should be provided by the national government. Ginners also vital in sustainability of cotton industry are under the jurisdiction of county governments. It cannot therefore be overemphasized that harmony and synchrony of functions between the two governments will be prerequisite to the sustainability of the cotton industry in Kenya. This disparity in functions was evident when we visited the Mwea County in Kenya and farmers informed us that support from the county may not be forthcoming because its cotton was not factored in the 2021 budget.

Cotton development authority

In 2006, the Government of Kenya established Cotton Development Authority (CODA) to promote cotton industry with the principal objectives to promote, co-ordinate, regulate and direct the cotton industry in Kenya. The core functions of CODA include to enforce regulations and standards as spelt out in the Cotton (General) Regulations, 2007; to promote cotton production through extension and advisory services; to strengthen research and technology development in the industry; to promote cotton value addition; and to facilitate marketing of cotton and its products (CODA, 2010). Two outstanding roles of CODA that need immediate attention are to enhance quality of cotton and promote cotton production through extension and advisory support.

Fibre crops development authority

In 2014, the Agriculture and Food Authority (AFA) was established under Section 3 of the Agriculture and Food Authority following the operationalization of the Crops Act 2013 that repealed the Acts Cotton Act, Cap 335 in 1990 and Sisal Industry Act, 1946 (No. 77 of 1946) of the parliament (CODA, 2021). Subsequently, the Fibre Crops Directorate was established and assumed the functions of the Sisal Board of Kenya and Cotton Development Authority. To date, the regulatory, development and promotion mandate of the fibre value chains is under the AFA-Fibre Crops Directorate (CODA, 2021; AFA 2022).

The functions of Fiber Crops Directorate are: provision of technical and advisory services; market research and dissemination of market information; promotion of product diversification and value addition; provision of technical support towards development of infrastructure in fibre crops; development of fibre standards and regulations along the chains in liaison with KEBs and the industry players; proposal of research in sisal and cotton in collaboration with institutions involved; and compliance with standards and regulatory framework for fibre crops and establishment of a reliable database of the fibre crops value chain (Fibre Crop Directorate, 2021).

Role of cotton ginneries in cotton industry

Data on number of cotton ginneries and functionality conflict a little bit. In 2010, CODA reported that there were 22 ginneries of which only 13 were operating (CODA, 2010). The 23 ginneries are located in six of the cotton growing regions of Kenya. The Eastern, Coast and Western regions each has five ginneries; Nyanza has six while Rift Valley and Central each has one (CODA, 2010).). In total, farmer cooperatives own 4 ginneries but only one is operational (CODA, 2010).

The 13 operational ginneries are also located in the six of the cotton growing regions of Kenya-Central (1), Eastern (3), Coast (3), Rift Valley (1), Western (1), and Nyanza (4) regions of Kenya (CODA, 2010). In 2017, Eleksie (3) reported that Kenya has 23 ginneries and only 8 were operational. Abuyeka (2021) listed eleven cotton ginneries currently in existence in Kenya, namely Gaitu ginnery (which is the largest), Luanda, Mpekoteni, Kitui, Malindi Ginneries Limited, Amukura Ginners Limited, Kitui Ginnery, Asego Holdings Limited, African Cotton Industries, Makueni Ginners Ltd., Meru Ginnery Limited, and Mpeketoni Ginnery (Table 1). It is notable that each of the cotton production regions in Kenya has at least one ginnery which is fair distribution given that the industry is

Table 1. Regional distribution of cotton ginneries in Kenya.

Region	Number of ginneries	Number operational CODA (49)	Eleven Ginneries Abuyeka (53)
1. Eastern	5	3	Kitui, Malindi, Makuweni. Meru
2. Coast	5	3	Mepketoni, Malindi
3. Western	5	1	Luanda, Amukura
4. Nyanza	6	4	Asego Holdings
5. Rift Valley	1	1	African Cotton Industries
6. Central	1	1	Gaitu Ginnery
Total	23	13	11

Source: This table was generated from text reported by CODA (2010) and Abuyeka (2021).

just being revived. However, effective and efficiency ginning of cotton is key to sustainable globally competitiveness of the textile industry in Kenya. CODA 2010, outlined several challenges and opportunities pertinent to cotton ginneries in Kenya. Key impediments to efficient functionality of ginneries included high operational costs due to use of old technology and frequent breakages, infrequent ginnery maintenance, low ginning outturn, high cost of power, below capacity operation and farmer owned ginneries have inadequate working capital for operations and weak management (CODA, 2010).

The Government of Kenya proposed several measures to improve efficiency of ginneries that include enhancing cotton production to enable ginners to work at full capacity, improving the management of farmer owned cooperative ginneries and concomitant resource mobilization; enhancing local and international partnerships in the sector; enhancing the ability of the sector to access bilateral soft loan and ginners to update ginning technology to international standards (CODA, 2010). Unlike the cotton textiles where some textiles have modernized there are no reports on adoption of modern technology in cotton ginning in Kenya. The constraint of inefficiency within the ginning section of the value chain still needs urgent attention.

Role of farmer cooperatives in the agriculture and potential of cotton farmer cooperatives

Agricultural cooperatives are agricultural-producer-owned with primary purpose to increase members' agricultural production and productivity achieved through facilitating access to finance, agricultural inputs, know-how, access to output markets and increased incomes (ATA, 2012). The role of agricultural cooperatives falls into four categories: to facilitate farmers' access to natural resources (land and water); to provide information, knowledge and extension services; to provide access to markets and productive assets such as seeds and tools and policy and decision making (SIFA, 2014). There are successful cooperative movements in Africa (the dairy sector in Kenya, coffee in Ethiopia, cotton in Mali and in Asia like Taiwan) that resulted in increased farmers' profits (Wang et al., 2019; Byjus; 2022; Vietnam (MoA&RD), 2018; Conroy, 2018) that are instrumental sector transformation. However, some cooperative movements have performed below expectation (Ruotsi, 1989).

Farmer cotton cooperatives in Kenya

Ruotsi 1989, reviewed the genesis of Cotton Cooperatives Societies in Kenya from mid-1970s, and understanding the genesis of their past challenges is key to strategizing the potential role in the Cotton Industry in Kenya. Cotton Cooperative Unions in Western Kenya took over from cotton ginneries in Western Kenya from their previous Kenyan-Asian owners in the 1970s. The main economic activity of the unions during the period, 1976-1984 was the ginning of seed cotton in their own ginnery and the related seed cotton buying activity. Additional activities undertaken by these unions are similar in all of them: they operated seasonal credit schemes with funds loaned from the Cooperative Bank of Kenya; operated farm input stores and owned lorries which transported seed cotton and the farm inputs.Their existence as economic units depended entirely on their performance as parts of the cotton industry (Ruotsi, 1989). Only one cooperative the Malaba/Malakisi Farmers Cooperative Union Ltd diversified and managed ginnery; the union also bought a soap plant and an oil mill.

Ruotsi 1989, gave three reasons for the decline in cotton production in Kenya in 1980 namely- perpetual delayed payment to farmers by the Cotton Lint and Marketing Board, low producer price for the seed cotton and poor performance of the Cooperative Societies and Unions, that served as agents of Cotton Board to buy and gin cotton, provide input credit to farmers. It is imperative that previous challenges of Cotton Cooperative Societies are identified and elucidated and measures put in place to eschew the previous impediments to cotton production in Kenya. The Government of Kenya has made reforms in the Cotton Cooperatives to address these past deficiencies through Cotton Development Authority (CODA).

Current status of cotton cooperative societies in Kenya

The Cotton Development Authority (CODA, 2010) reported that the Government has written off all non-performing loans owed to the Cooperative bank of Kenya by the societies in the tune of US $ 2 million US $ in 2007/2008 (49). However, the Cooperatives Societies still lacked capital, management skills and good governance. The Authority has embarked on a programme to train groups in collaboration (CODA, 2010). The revival of Cooperative Societies is imperative to strength farmers' bargaining power. It is a welcome venture but it cannot be overemphasized that the preceding challenges if not addressed will erode the confidence of Banks in providing loan to farmers' cooperative and also the farmers' confidence in cooperative societies. The Government of Kenya has also embarked on restructuring Kenya Textiles Industry.

The structure of the cotton, apparel and textiles industry in Kenya

The Cotton, Textiles, and Apparel (CTA) industry is Kenya's second-largest manufacturing industry second to food processing. It is core industry to Kenya economic development (Anon 2021; KIA, 2016; Onyango et al; 2009) and has potential to play a key role in anchoring spurring into middle income status (Gok, 2015). Kenya's Textile Industry consists of three main segments- Textile mills, Textile product mills and Apparel manufacturing (Matheka, 2021). Textile mills provide the raw material to make apparel/clothing and textile products, Textile product mills convert raw textiles into finished products, other than apparel such as carpets, rugs, towels, curtains, and sheets while Apparel manufacturing transforms fabrics produced by textile manufacturers into clothing and accessories (Matheka, 2021). Alternatively, the textile industry could broadly be broken into yarn spinning, fabric manufacturing and garment manufacturing. Kenya has 52 textile mills with only 15 currently operational and operate at less than 45% of total capacity (Ikiara and Ndirangu, 2003). Many of the existing mills use outdated technology and the sector suffers from low levels of labor skills and low productivity. The top 10 best textiles industries in Kenya are Spin Knit Limited, Rivatex East Africa Limited, Thika Cloth Mills, Sunflag Textile and Knitwear Mills: Spinners and Spinners Ltd, Fine Spinners Ltd, Alliance Garment Industries Ltd, KEMA East Africa Ltd, Supra Textiles Ltd and Specialized Towel Manufacturers Ltd (Abeyuka, 2020; Rivatex, 2021; Anon, 2021; Malicha and Njoroge, 2020). The Government of Kenya made commendable progress in supporting revitalization of the Rift Valley Textile Industry (Rivatex), which has transformed from outdated technology, low levels of labor skills and low productivity (Ikiara and Ndirangu, 2003; CODA, 2010) to the current state-of-art in textile technology.

Rift valley textile industry

In 2007, Rift Valley Textile Industry (Rivatex) was acquired by Moi University, but prior to this, the company had old and outdated technology machines which could not compete internationally to realize high returns (Rivatex, 2022**).** Through intervention of Government and facilitation of credit the textile has undergone extreme transformation of the vertically integrated textile mill; today it has highly trained staff and is ranked the best facility for woven fabric in East Africa. The presence of highly efficient equipment has opened doors to students from institutions of higher learning and those from Technical Vocational and Training (TVET) colleges who now have an opportunity to interact and gain practical skills from the new textile technology (Rivatex, 2022**).** The textile has the current state-of- the-art machines at the facility. Spinning, weaving and processing sections are fully equipped with ultra-modern machines thanks to the Government's unwavering support to ensure textile industry is fully revived and contributes to the country's Gross Domestic Product (GDP) as well as create employment. Currently, the facility produces 40,000 meters of finished fabric daily with a capacity of producing more than 100,000 meters per day, depending on the demand along with an increase in the number of spindles from the initial 500 to 16,800. Initially, the facility used to consume less than 10,000 bales of cotton annually but the provision of state-of-the-art machines has seen a tremendous increase that will require more than 40,000 bales annually sourced from farmers through ginneries. With the commissioning of the new machines, the company has been able to take up huge orders from the Government such as supplying superior fabrics for the National Police Service, County hospitals, Kenya Power (KP), Geothermal Development Company (GDC), among many others (Rivatex, 2022).

Modernization of the factory has significantly increased the production capacity and diversification of product range and outfits in the cloth line that are very attractive, targeting both local and global markets. The faculty has invested in quality assurance and control, which ensures all the products are fully tested and checked to meet international standards. The modernization of the factory process has encouraged counties to start cottage industry and Small Medium Sized Enterprises (SMEs) who complete the value chain by adding value to our products (garmenting and tailoring) while creating employment in the forward integration (Rivatex, 2022).

Towards production of certified quality cotton seeds supply in Kenya

For long time cotton farmers in Kenya have been growing

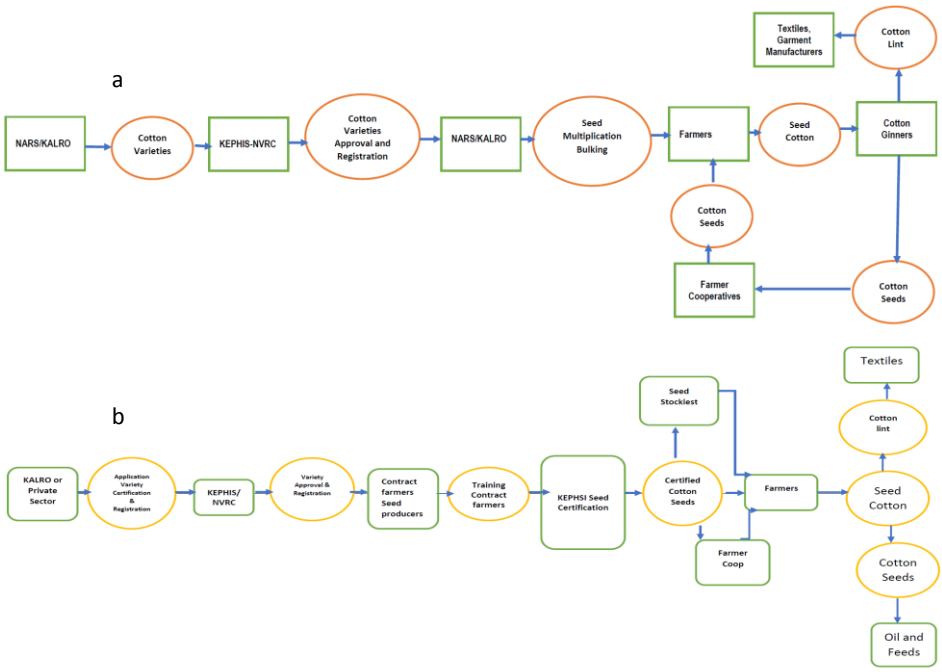

Figure 3. (a) Old pathway for supply of cotton seeds to small holder farmers and (b) pathways for conventional certified cotton seeds in Kenya.
Source: These figures were developed by authors from information obtained from the research using Microsoft Word 10 icon "Shapes

uncertified cotton seeds left after ginners separate seed cotton into lint and cotton seed (CODA, 2010). The cotton seeds are retained by ginners and distributed to farmer directly or through farmer Cooperatives Societies (Figure 3). As a result of continued recycling of cotton seeds from the ginners, the varieties slowly degenerate in purity and quality. Degeneration of cotton varieties is expected because although cotton is predominantly self-fertilizing, in the presence of pollinators outcrossing 10-48% have been reported, causing gradual decline in seed purity. When farmers grow uncertified cotton seeds they cannot be globally competitive and will not realize optimal production even with the use of best agronomy practices including fertilizers and pest control. Many experts agree that timely, availability quality certified seeds at affordable prices are critical to enhance agricultural production in Africa and cotton is no exception (CODA, 2010). Diverse national, regional and international organizations are working in Africa to address the issue of providing quality seeds to farmers including USAID, AGRA, AFSTA, Kenya Seed improving food security, resilience, income of livelihoods for smallholder farmers in Trade Association, Wageningen University, Africa Seed Trade Association, Kenya Seed Trade Association among others (Europeanseed, 2019, AFSTA, 2021; AGRA, 2021; USAID, 2020; USAID, 2017; Kenya Seed Trade Association, 2021; Access to Seed Index, 2019). It cannot be overemphasized that farmers' access to quality seeds is the first and important starting point toward meeting Africa's continental and individual country for realizing agricultural goals such as food security, nutritional security, improved household incomes, improved livelihoods among others.

In 2008, the Government of Kenya recognized that to revitalize the cotton industry calls for timely availability of quality and high yielding cotton varieties for farmers (CODA, 2010). Key tenets of realizing this goal call for maintenance of genetic purity of introduced, promising and existing commercial varieties. Towards this end, the government embarked on evaluation of newly introduced varieties from Monsanto and anticipated to get drought tolerant and insect resistant Bt varieties from India and China. In addition, in 2009, the production of basic and foundation seed of the two conventional commercial varieties HART 89M and KSA 81M Kenya Agricultural Research Institute (KARI) centers in Kibos and Mwea was enhanced to maintain genetic purity (CODA, 2010) (Figure 3b). In 2009, a total of 150 metric tonnes of certified seed was produced by farmers who had been contracted by KARI from the Coastal Region. This pathway to supply certified quality seeds, though good, still has constraints such as inadequacy capacity in seed bulking, needs more trained manpower and financial support to purchase equipment for the commercial production of seed by the private sector (Gitonga et al, 2016). Given the high demand of quality certified cotton seeds in Kenya and given that the Government encouraged the farmers to source for different improved

cotton sees, it is imperative that these limitations are alleviated and to enhance supply of certified quality cotton seeds from the public and private sector.

However, in 2019, Kenya approved commercial release of improved insect resistant and hybrid cotton seeds. This calls for the need for alternative options but complementary system that allow timely supply of quality certified cotton seeds to farmers. Private seeds producers have to recoup input costs and some profit margin is needed.

Toward improved quality cotton seeds and opportunities for sustainability

One solution to alleviate the supply of quality seeds is for farmers to adopt the improved insect resistant hybrid cotton seeds approved for commercialization in Kenya in 2019 under several jurisdictions-the Biosafety Act 2009 (KNBA, 2021a), Regulations for Environmental Release (15), Kenya Seed Act of and National Performance Regulation of The Kenya Seeds and Plant Varieties (Amendment) Act, 2012 (2013) and Seeds and Plant Varieties (National Performance Trials) Regulations, 2009 (Gok, 2009; 2012b).

Based on field trials, environmental and food safety research, in December 2019, Kenya approved BollGuard II Bt. cotton from the Mahyco Seeds Company in India for commercial release in Kenya. Three “Essentially Derived Varieties“ of insect resistant hybrid cotton varieties have been registered by KEPHIS designated as- MAHYCO C 570 BGII, MAHYCO C 569 BGII, MAHYCO C 571 BGII and four conventional hybrids cotton varieties designated as- MAHYCO C 567, MAHYCO C 569, MAHYCO C 570, MAHYCO C 571 (KEPHIS, 2021a, 2022). Mahyco commercialized this technology in collaboration with various government agencies. Given that the Bt cotton hybrid technology is technology to Kenyan farmers and seed prices are higher, farmers may recycle seeds unless they are supported technically and financially. Therefore, the government initially purchased Bt cotton hybrids seeds and supplied them free to farmers for planting on-farm demonstration plots in various cotton growing regions.

As a launching phase, in 2020, the Kenya Government through Agriculture and Food Authority (AFA) purchased 700 kilos of Bt cotton seeds of three hybrids (C569, C570 and C571) from Mahyco. About 500 farmers in Western and Coastal regions of Kenya received free seeds to plant in demonstration plots (Figure 1). For the next growing season from October 2020 to January 2021 (short rain season), the government purchased additional 16 metric tons (16,000 kilos) of Bt cotton hybrid seeds from Mahyco and expanded the demonstration plots with 10,000 acres in 9 counties in Eastern and Central Kenya.

The demonstration plots showcased the value of the Bt cotton and hybrid technology as well as training of farmers on agronomic and crop management practices associated with Bt cotton hybrid technology. Observing the Bt cotton demonstration and subsequent results, a farmer in Central Kenya area expressed “…*the remarkable progress indicated so far has convinced me to invest in Bt cotton seed. I will not wait to receive the seeds from the government. I hope the seeds can be available in my local farm input suppliers (agrovet) so that I can purchase, just like we get hybrid maize seeds”* (Alliance for Science, 2021). With the success of demonstration trials, farmers’ demand for Bt cotton hybrids are expanding.

But due to the financial crunch faced by the government because of the COVID-19 Pandemic, the purchase of Bt cotton seeds from Mahyco Company in India has been hampered. Therefore, a transition from subsidy to farmers independently purchasing Bt cotton seeds in a few years’ time is the preferred approach. In the 2021 growing season (March/April 2021) when seed was no longer subsidized, only 10% of farmers in a cotton growing areas of 37,000 acres purchased improved Mahyco’s Bt cotton seeds.

Options for sustainable seed supply to farmers in Kenya

The free supply of Bt cotton seeds is not a sustainable and viable option in a long run. Due to the issues related to foreign exchange and financial constraints faced by the government due to the COVID-19 Pandemic, the Kenyan government is not able to purchase seeds for 2021 growing seasons. The government, therefore in collaboration with the technology provider (Mahyco Company), is under discussion to develop a strategy and options for providing access to Bt cotton hybrid seeds.

Based on the discussion with various stakeholders, the following four options were suggested as potential pathways to provide access to Bt cotton hybrid seeds to farmers. Given that the farmers have limited access to credit to purchase Bt cotton seeds, the government and the technology provider will need to work together to develop viable and affordable schemes to support the cotton sector. Stakeholders are of the opinion that diversifying options of improved cotton technologies to include Bt cotton hybrids, non-Bt cotton hybrids and even open pollinated ones would improve access to seed and satisfy the diverse needs of farmers. Such diverse interest can also have challenges in the current setting of the cotton seed system that is not well established. Therefore, possible options for consideration and further discussions are as follows.

Option 1: Federal/National government seed purchase subsidies

This option would enable farmers to purchase seeds at

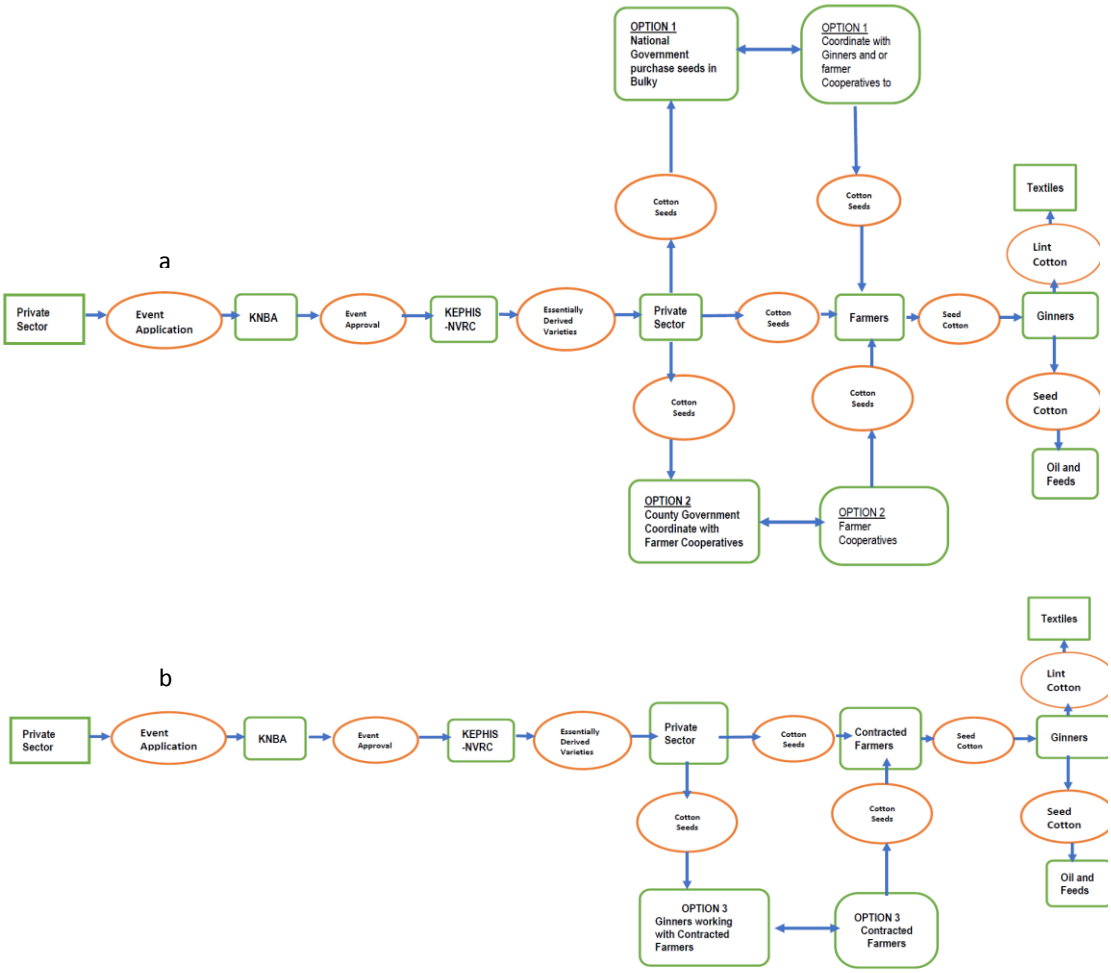

Figure 4. Intermediary hybrid Bt cotton seed systems for provision of approved quality Bt cotton hybrids seeds to smallholder farmers in Kenya.
Source: These figures were developed by authors from information obtained from the research using Microsoft Word 10 icon "Shapes.

discounted prices for several years to enhance wider adoption of the technology and empower farmers with their own financial resources. The government would need to make capital/financing arrangements through national and international instruments (Figure 4a).

This strategy would include government purchase of Mahyco seeds. Farmers would purchase the seeds at a discount for three years, gradually transitioning to paying full costs in the fourth year. The discount could follow this schedule to purchase seeds from cooperatives, ginners or agro-dealers: Year 1 (2021) – 75% discount on the Bt cotton seed costs; Year 2 (2022) – 50% discount on the Bt cotton seed costs; Year 3 (2023) – 25% discount on the Bt cotton seed costs.

As a part of this strategy, the government could collaborate with cooperatives and ginneries to recover the seed costs through a levy-based system where farmers are charged certain portions of the selling price when they bring their harvested cotton for sale. This would require agreement with government, ginneries, and farmers on the levy rates and payback period.

Option 2: Use of farmers' cooperatives and county government in cotton seed supply

Farmers' cooperatives and county governments could play a significant intermediary role in the supply of improved certified cotton seeds to cotton farmers either individually or synergistically (Figure 4a). Today, Kenya has about 20 cotton operational Farmer Cooperatives societies each with about 200 members. In addition, the government has taken measures to revitalize and improve governance standards of the cooperatives, established annual general meetings and financial accountability measures such as annual account audit. It is also notable that in 2010, Kenya adopted a new constitution that established two arms of government- the National and

County Governments with devolved functions. The provision of agricultural services to small holder farmers is under the mandate of county governments.

In option 2, the County Governments could directly purchase cotton seeds in bulk from the private sector such as Mahyco thus ensuring steady and regular supply of improved seeds. The advantage of bulky purchase is to give the private sector the impetus to supply seeds regularly and on annual basis, seed could be sold at discounted rates. These seeds could be channeled to individual county farmers directly or more efficiently through farmer cooperatives. For example, in 2020, Lamu County government purchase improved certified seeds directly from the Mahyco Seeds Company in India and provided them to local Lamu County farmers. In 2021, Lamu County government ordered 4.5 metric tons (4,500 kilos) of hybrid Bt cotton seeds from Mahyco for its farmers. It is anticipated that the order of improved seeds by Lamu County and other counties in Kenya will grow yearly.

Cotton growing counties need to put the cotton sector high in their annual development agenda and include the sector in development plans and budgetary allocation. Although County governments receive their annual development budgetary allocation from the National Government and it is incumbent upon them to provide budgetary allocation to the cotton sector. Unlike Lamu County which purchased cotton seeds for its farmers, one of the counties we visited could not support in purchasing improved hybrid Bt Cotton seed because in 2021 they had not included cotton sector in their development agenda and budgetary allocation.

The advantage of County Government working in collaboration with farmer cooperatives is that the later have registered farmers, have mechanisms for providing seeds to farmers and ways of recovering debt in case seeds are given on credit (Figure 4a). The Government through Cooperatives could work on debt recovery procedures for farmers but seeds on credit. This option could also enable increased farmers' access to credit and allow cooperatives to purchase seeds from Mahyco and supply farmers at a discount rate. One limitation with this option is that the number of farmers covered by the cooperatives scheme could be less than with other options.

If counties work directly with farmers then they must find way of registering farmers and clustering them in groups. It is also possible for several countries in Kenya to cluster together and purchase seeds in bulk. This will give the private sector the needed economies of scale to produce adequate seeds in a timely manner. The discounts, the levy rates and payback period can best be established between farmers and the cooperatives and with the involvement and support of the government. In major cotton producing counties in Kenya, county governments can also form "county - clusters" of cotton farmers and develop a mechanism of purchase of seeds through tripartite partnerships between county government clusters, farmer cooperative, and ginners.

Option 3: Contract farming and ginneries support in supply of cotton seeds

In option 3, ginners could play an additional major role in cotton seed supply during the transition intermediary period. This will be modification of the old model where ginners used to supply recycled cotton seeds to farmers as shown in Figure 3a; they should supply quality certified cotton seeds (Figure 4b). Through government support such as extension servicers, the farmers can be organized to work in partnership with ginners under contract. The ginners could purchase Bt cotton hybrid seeds directly from Mahyco and supply seeds to farmers through a post-harvest payback agreement scheme. The details of the payback arrangement can be worked out in the contracts with farmers (Figure 4b). The practicability of this option is exemplified in 2021 growing season, where some ginners purchased 1.2 metric tons of Bt cotton hybrid seeds from Mahyco and requested for an additional 700 to 800 kilos of seeds to meet their demand. This method can also be reinforced in future to include the ginners supplying other enhancing production inputs such as pesticides, fertilizers and credit finance to farmers. The purchase of Bt hybrid cotton seeds by farmer cooperatives, ginners, and county governments directly from Mahyco is expected to grow in 2022.

Option 4: Selling seeds to farmers through open market scheme

For future sustainability supply of improved cotton seeds to farmers will be needed for farmers to be full established in cotton production and purchase seeds from the open market without government subsidies. Farmers will directly purchase seeds or buy though Cooperatives or Agro-dealers (Figure 5). In addition, the seed companies will need to establish seed production centre in Africa that may serve several countries that have commercialized Bt cotton such as Ethiopia, Malawi, Nigeria and many that may come on board in future to take advantage of economies of scale (Alliance for Seed, 2020; 2021). Today, Kenya has agro-dealers actively selling maize and other crops seeds, pesticides, fertilizers and other inputs. In Nigeria and Malawi, Bt cotton hybrid seeds are supplied through agro-dealers. The open market option can be viable and competitive but will require a strong cotton seed quality regulatory oversight by the government to address the issues related to fake seeds. This can be addressed through enforcement of policies related to strict seed quality.

It is notable that there will be co-evolution of supply of certified cotton in Kenya to small holder farmers. At the commencement the key players will be county governments, ginners, farmers cooperatives working

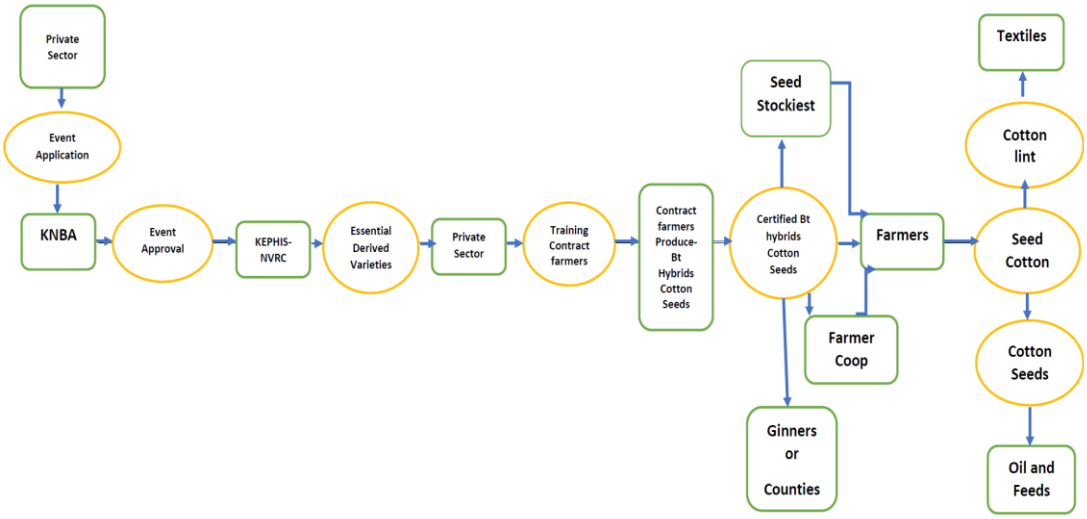

Figure 5. Future alternative sustainable hybrid Bt Cotton Seed Systems for provision of approved quality and adequate seeds to small holder farmers in Kenya.
Source: This figure was developed by authors from information obtained from the research using Microsoft Word 10 icon "Shapes

synergistically or independently with private sector to supply quality seeds to farmers. With the government playing a key regulatory role these options will eventually crystallize into the private sector consisting of seed companies and agro-dealers, that provide quality supply certified cotton seeds to farmers. Farmers' cooperatives persist to enhance farmers' productivity if they maintain good accountability and management. The later also depends on the government support and regulatory oversight.

As Kenya embarks on revitalizing the cotton Industry, it is notable that there are great opportunities for the country to tap into the global market of textiles which the country has not fully utilized including but not limited to African Growth and Opportunity Act (AGOA) ACP-EU Trade Agreement Common Market of Eastern and Southern Africa (COMESA) and the East African Community (EAC) (AGOA, 2021).

Lessons learnt from Indian's experience in the deployment of Bt cotton

India adopted Bt cotton technology in 2002 when the area under cotton production was only 50,000 hectares. In 2015, the area increased to 12.2 million ha (of which 95% or 11.6 million ha was bt cotton) and an output of 39 miilion bales, overtaking China and becoming the largest producer of cotton globally (Arora and Bansal, 2012; James, 2015). In 2020/2021, the area under cotton production was projected to be 12.9 million ha (GAIN, 2021). Other countries that commercialized the Bt technology include Nigeria, Kenya, Malawi, Ethiopia, USA, South Africa, Mexico, and China, after thorough risk assessment of food safety and environmental safety and socio-economic considerations (Mexico-BCH-CBD, 2017, a, b, d; Nkechi, 2020; South Africa BCH-CDB, 2015). Today about 95% of cotton grown in India is Bt and is grown by small holder farmers. There are important lessons to be learnt from India's experience: need to have a functional biosafety regulatory system, diversify released Bt hybrids, expand the Bt hybrid seed technology providers, inclusion of refuge cotton and addressing challenges of seed pricing (Arora and Bansal, 2012).

Mahyco commercialized three Bt cotton hybrids in India in 2002 and later sub-licensed the Bt technology to India companies resulting in increase in the commercialization of 20 Bt hybrids in 2005, 131 in 2007 and 207 in 2008. This helped the farmers to get the suitable varieties for different ecological zones. It one factor that facilitated changing India's biosafety approval systems based on food safety and environmental from case-by-case approval to "Event based" (Arora and Bansal, 2012) and separation of "Event Approval from National Variety Release and Registration processes (Olalekan et al., 2021). Bt cotton seed pricing is a concern and has been reviewed several times by the state and central government for resource poor farmers to better access technology. Arora and Malik 2019, argue that price control should be balanced so as not to disincentive private sector investment in innovation. They propose the use of complementary approaches such as government reduction in regulatory approval costs, simplification of regulatory approval process subsidy for R and D among others (Arora and Malik, 2019).

There are reports of GM crops genetically engineered to produce insecticidal proteins Bt showing re-emergence

of resistant (Bruce and Carrière, 2019; Arora and Malik, 2019), re-emergence of Pink Bollworm globally, in USA, India, Mexico (Storer et al, 2010; Dhurua and Gujar, 2010; Jurat-Fuentes et a, 2003; Rocha-Munive et al, 2018). In United States, farmers delayed resistance by planting non-Bt cotton refuges from 1996 to 2005; they cooperated in a program that used Bt cotton, mass releases of sterile moths, and other tactics to eradicate this pest from the region. In China, farmers reversed low levels of pink bollworm resistance to Bt cotton by planting second-generation hybrid seeds from crosses between Bt and non-Bt cotton. 25% non-Bt cotton plants were randomly interspersed within fields of Bt cotton. In India where non-Bt cotton refuges are scarce and pink bollworm resistance to pyramided Bt cotton producing Cry1Ac and Cry2Ab toxins is widespread and integrated pest management emphasizing shortening of the cotton season, destruction of crop residues, and other tactics is now essential.

Global opportunities for sustainability of cotton industry

There are global and regional opportunities that could spur the cotton industry in Kenya through creating ready market for the manufactured textiles. They are African Growth and Opportunity Act (AGOA), ACP-EU Trade Agreement, African Continental Free Trade Area, Common Market of Eastern and Southern Africa (COMESA), ACP-EU Trade Agreement and East African Community (EAC).

African growth and opportunity act

The African Growth and Opportunity Act (AGOA) is a United States Trade Act, enacted on 18 May 2000 as Public Law 106 of the 200th Congress. AGOA has since been renewed to 2025. (AGOA, 2021). The legislation significantly enhances market access to the US for qualifying Sub-Saharan African (SSA) countries. The preferential trade agreement with the U.S. Government temporarily eliminated all duties and quotas on Kenyan textile exports to the U.S. market until the year 2015. As a result of this agreement, which took effect in 2001, Kenya's textile exports to the U.S. have increased significantly over the past decade. However, most of Kenya's textile industry inputs continue to be imported (AFDB, 2013, World Bank 2004). Due to the minimal benefits captured by cotton producers. The World Bank, 2005 identified that a necessary condition for "gearing up the industry" is to improve the coordination and linkages throughout the cotton sector's value chain.

African, Caribbean and Pacific-EU trade agreement

Economic Partnership Agreements (EPAs) are trade and development agreements negotiated between the EU and African, Caribbean and Pacific (ACP) countries and regions (European Commission, 2021). EU's trade relationship with ACP countries is stipulated by the Cotonou Partnership Agreement signed in 2000 (European Commission, 2021, EU, 2022). This comprehensive political, economic and development partnership was due to expire in 2020 and Parties have negotiated a successor agreement (the so-called 'Post-Cotonou Agreement'), which was initialed by the chief negotiators on 15 April 2021 (European Commission, 2021). In Sub-Saharan Africa, there are currently 14 countries implementing an EPA with Kenya (Embassy of Kenya Brussels, 2022). EPAs with Sub-Saharan African countries and other EU free trade agreements with Northern African countries contribute to AfCFTA and to the long-term perspective of a continent-to-continent free trade agreement (European Commission, 2021).

Common market of Eastern and Southern Africa (COMESA)

Kenya is a member of COMESA, a regional economic co-operation organization, which has been working to reduce trade barriers applied to goods produced within and traded among its 19 member countries. COMESA's current strategy can thus be summed up in the phrase 'economic prosperity through regional integration'. With its 21 Member States, population of over 583 million a Gross Domestic Product of $805 billion, a global export/import trade in goods worth US$ 324 billion, COMESA forms a major marketplace for both internal and external trading. Geographically, COMESA is almost two thirds of the African Continent with an area of 12 million (sq km) (COMESA, 2021a). COMESA strategy has three priority areas-Free Trade Area has been in effect since 2000. COMESA has three priorities- a Free Trade Area (PTA), Customs Union, Trade Promotion (COMESA, 2021b). The FTA was achieved on 31st October 2000 when nine of the member States namely eliminated their tariffs on COMESA originating products, in accordance with the tariff reduction schedule adopted in 1992. Today eleven COMESA countries Djibouti, Kenya, Madagascar, Malawi, Mauritius, Sudan, Zambia, Zimbabwe, Rwanda and Burundi have eliminated customs tariffs and are on the eventual elimination of quantitative restrictions and other non-tariff barriers (COMESA, 2022).

East African Community (EAC)

The East Africa Community has four integration Pillars viz, Customs Union, Common Market, Monetary Union, and Political Federation (EAC, 2022). Customs Union was the first Regional Integration milestone and critical foundation of the East African Community (EAC) and

came into force since 2005 (EAC, 2021b). Under this pillar Partner States agreed to establish free trade (or zero duty imposed) on goods and services amongst themselves and agreed on a common external tariff (CET), whereby imports from countries outside the EAC zone are subjected to the same tariff when sold to any EAC Partner State (COMESA, 2022). The Common Market was the second Regional Integration milestone of the East African Community (EAC) and came into force in 2010. Under this pillar EAC Partner States maintain a liberal stance towards the four Freedoms of movement for all the factors of production including Free Movement of Goods (EAC, 2021c). The EAC comprises Kenya, Tanzania, Uganda, Rwanda, Burundi, South Sudan.
Kenya being a member makes the country access market population of approximately 177 million across Seven (International Trade Administration, 2021) and Democratic Republic of Congo will soon join EAC.

African continental free trade area

African Continental Free Trade Area (AfCFTA) is a flagship project of Agenda 2063 of the African Union (AfCFTA, 2021a), approved by the 18th ordinary Session of Assembly of Heads of State and Government, held in Addis Ababa, Ethiopia in January 2012 which adopted the decision to establish a Continental Free Trade Area (AfCFTA, 2021a). The AfCFTA aims to accelerating intra-African trade and boosting Africa's trading position in the global market by strengthening Africa's common voice and policy space in global trade negotiations. To date, the Agreement has been signed by 54 out of 55-member States and by February 5, 2022, 36 countries ratified the AfCFTA agreement. It creates a single market for goods, services, facilitated by movement of persons in order to deepen the economic integration of the African continent and in accordance with the Pan African Vision of "An integrated, prosperous and peaceful Africa" enshrined in Agenda 2063. The cooperation is expected to benefit over 1.2 billion people, protect the national interests of the 55 AfCFTA nations and the safety and wellbeing of their citizens. The first went into effect in January 1, 2021 (AfCFTA, 2021b). Kenya was among nearly 55 African nations that signed a deal to create the AfCFTA in Kigali, Rwanda, on March 21, 2018. AfCFTA covers a population of 1.3 billion from 55 countries with GDP valued at US$3.4 trillion (Silk Road Briefing, 2021).

CONCLUSIONS AND RECOMMENDATIONS

Institutional reform

The Government of Kenya has made diverse reforms in institutional framework with great potential to enhance the functionality of cotton value chain including establishment of Fibre Crops Development Authority, Cotton Development Authority, Kenya National Biosafety Authority, Textile Industries. These reforms are quite appropriate and if they institutions perform their functions adequately this will go a long way in enhancing them.

Reforms in the policies and regulatory framework

The Government has done an excellent work in policy reforms covering such as the Presidential Four Pillars, Kenya Vision 2030, The Co-operative Societies Act (Cap 490 of the laws of Kenya, Cotton Act, Cap 335 in 1990, Biosafety Act 2009 and Implementing Regulations. The polices if operationalized and laws enforced would spur growth and sustainability of the Cotton Industry in Kenya. KEPHIS has exemplified adequate institutional and human capacity to provide oversight over Biotech products after NBA Biosafety approval until the farmer gets quality, certified seeds.

Reforms in the seed system

Supply of timely, adequate and affordable cotton seeds, a very important segment of the cotton value is a weak link that needs immediate attention. Given both the Bt technology and hybrid cotton technology are new to farmers in Kenya, the continued engagement of government and technology providers to empower farmers and institutions that support farmers is critical in four ways. First, the County Governments should play additional role in engaging cotton producing counties in the different provinces in Kenya to form cotton producing 'county-clusters' to support access to Bt cotton hybrid seed and related inputs and access to cotton/product market to encourage cotton producing smallholders. Second, the four options to access Bt cotton hybrid seeds-Government Supported Scheme, Contract Farming Scheme and Open Market Scheme need to be further discussed with the government, key stakeholders and technology providers. Third, the hybrid cotton technology is new to Kenyan farmers and requires special agronomic treatment, crop management practices including fertilizers and irrigation use. Extension services and training of farmers and other relevant stakeholders in agronomy, crop management and product stewardship should be an integral component of commercialization and scaling-up strategy for Bt cotton. Fourth, the long-term strategy should be to develop innovative partnerships to produce Bt cotton hybrid seeds locally or regionally in Kenya or in East Africa.

Important role of county governments

Because of the developed agricultural functions, it is

important that 23 of the cotton growing counties embark on intensified production of cotton in their respective areas. The participation of the country to enhance cotton production is one weak link in the cotton value chain. Key ways of providing support are through extension service, supporting farmer cooperative societies in enhancing the financial and administrative function of cooperative, ensuring stockiest supply of genuine farm inputs including seeds, chemical input among others.

Farmer cooperative societies

Cooperative societies have a very vital role in strengthening farmers' cooperatives but need a lot of strengthening in the following ways: Establishing of cotton farmers' cooperatives in countries that have none, and ensuring good administrative and financial accountability of these cooperatives.

Opportunities in the global and regional market

There are opportunities in the global and regional markets for textiles including the - African Growth and Opportunity Act (AGOA), ACP-EU Trade Agreement, Common Market of Eastern and Southern Africa (COMESA), ACP-EU Trade Agreement and East African Community (EAC) that should be exploited.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessment of *Exserohilum turcicum* using molecular markers for sustainable maize production in Tanzania

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The *Exserohilum turcicum* fungus causes the Northern leaf blight (NLB) of maize all over the world. In Tanzania, this disease is considered as a major foliar disease with yield losses of 23.9 to 62.8%. To understand the genetic variations of *E. turcicum* from maize fields in Tanzania, a survey was conducted in four regions, including Morogoro, Iringa, Njombe, and Mbeya. Leaf samples were collected from diseased plants exhibiting NLB symptoms. Using aseptic technique, pure colonies of fungal pathogens were isolated on selective media followed with molecular analysis. The internal transcribed ribosomal DNA (ITS), six microsatellite markers of simple sequence repeat (SSR) nature, and two specific mating type primers (MAT) designed to amplify *MAT1* and *MAT2* gene sequences were used in this study. With ITS marker, 14 isolates were amplified, Sanger sequenced, and their sequences were deposited to GenBank with accession numbers MT124699-MT124712. The SSR results were scored using base pairs in each genotype and subjected to power marker software to determine genetic variations. A total of four alleles across two loci were detected, with gene diversity (0.26 - 0.58). Polymorphic bands revealed 4 and 5 genotypes using SSR 06 and SSR 024, respectively. The polymorphic information content (PIC) of SSR 06 and SSR 024 loci were 0.25 and 0.52, respectively. The specific MAT markers demonstrated dominance of *MAT 2* over *MAT 1*. The distribution of *MAT 1* and *MAT 2* in humid highlands of Mbeya, Njombe, and Iringa regions indicated possibility of sexual reproduction and good potential for sexual recombination than in the dry lowland of Morogoro region. This study is the first report on genetic diversity of *E. turcicum* in Tanzania, further studies using robust molecular and sequencing techniques is imperative in Tanzania.

Key words: *Exserohilum turcicum,* maize, microsatellite, mating type primers, ITS primers, Tanzania.

INTRODUCTION

Exserohilum turcicum (Pass.) Leonard and Suggs (1974) of maize is an important foliar disease in Tanzania (Dong et al., 2008; Nwanosike, 2016). The disease is potentially distributed all over the world (Ramathani et al., 2011). The pathogen also infects sorghum and other related grasses (Harlapur et al., 2007; PANNAR Seed Company, 2009); however, epidemic may be sporadic depending on the environment and varieties to resist infection (Degefu,

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1990).

This disease is a threat to maize production globally (Muiru et al., 2010; Levic et al., 2008; Nwanosike et al., 2017). In Tanzania, yield losses ranged from 23.9% in Morogoro to as high as 62.8% in Mbeya (Nwanosike, 2016). Population studies on the pathogen become imperative to provide understanding of the genetic diversity (Nei, 1973; Ferguson and Carson, 2007; Haasbroek et al., 2014; McDonald and McDermott, 1993).

The studies of *E. turcicum* genetic diversity in various countries concentrated on use of RAPDs, AFLP, and isozymes (Dong et al., 2008; Muiru et al., 2010), but microsatellites markers considered as choice markers for population studies (Chambers and MacAvoy, 2000; Ellegren, 2004). However, SSR has been used primarily to detect polymorphism amongst the parental cultivated varieties (Klein et al., 2000). Ferguson and Carson (2004) reported that the population genetic structure of *Setosphaeria turcica* is subjective to the result of both sexual and asexual reproduction. Although the sexual phase of *E. turcicum* has not been discovered in the field, evidence of recombination exists especially in tropical populations (Borchardt et al., 1998b).

Simple sequence repeats are sections of DNA repetitive units (2-6 bp in length) inside all eukaryotic organisms (Quellar et al., 1993). Identification of nucleotide sequences in the adjoining regions of the microsatellite facilitates development of specific primers (generally 20-25 bp) to amplify the SSR by polymerase chain reaction. Ramathani et al. (2011) reported the type of mating primers that amplified the *MAT1* and *MAT2* idiomorphs of *E. turcicum* with fragment size of 154 and 197 bp, respectively. The efficacy of such primers was used to test maize and sorghum derived *E. turcicum* in Uganda (Ramathani, 2010). However, in South Africa, Haasbroek et al. (2014) designed and developed mating types idiomorphs of the pathogen with higher fragment size for mating type 1 and mating type 2, which was used to screen fungal isolates of maize and sorghum. It has a single locus and two-allele mating system and as such exhibits two forms of mating type (Borchardt et al., 1998a; Klix et al., 2010).

NLB is a destructive foliar disease in maize growing areas of Tanzania and is reflected as a limiting biotic factor hampering successful cultivation of maize. Despite efforts of the poor resource for farmers to improve yield, it remains low 1.3 to 1.5 tons/ha (Nkonya et al., 1998). Therefore, this study will apply SSR loci and species-specific primers designed based on internal transcribed ribosomal DNA (ITS) and 5.8s rDNA sequences to determine genetic diversity and mating types of Tanzania isolates, respectively.

MATERIALS AND METHODS

Leaf samples showing lesions were collected in 38 fields during a survey in 2012 to 2013. Twenty-eight (28) samples were collected from Southern highland regions including Iringa (10), Njombe (8), and Mbeya (10), and ten samples from Eastern region (Morogoro), using the Global Positioning System. Samples were surface sterilised and incubated for 2 days at 25±3°C, in laboratory. The fungus were isolated on V8 agar medium (Harlapur, 2005). *E. turcicum* cultured in a liquid media, the mycelia were collected and preserved at 4°C (Muiru et al., 2010).

DNA was extracted using Cetyltrimethylammonium bromide (CTAB) protocols by Moller et al. (1993) and adapted by Muiru et al. (2010). The quality and quantity of extracted DNA was measured on NanoDrop Spectrophotometer (Thermofisher, Waltham, MA) at ratio of 260/280 wavelength. The extracted DNA was stored at -20°C for use in PCR experiments.

PCR reactions and cycles

The identity of the isolates was confirmed through PCR sequence information from product targeting of the internal transcribed spacer 1 and 2 (ITS) of the 5.8S ribosomal RNA region using specific primers that were previously used by Ramathani (2010) (Table 1). The PCR amplicons were Sanger sequenced and blasted to the NCBI GenBank to identify isolate with more hits to our sequences.

Six microsatellite primers (Table 2) obtained from several related loci of ascomycetes and two specific MAT markers; *MAT1* and *MAT2* (Table 1) were used to test for polymorphism of 20 *E. turcicum* maize isolates using the protocol described by Ramathani (2010) and Ramathani et al. (2011), with amendments.

Amplifications were performed in a thermo cycler (Gene Amp PCR system 9700 Applied Bio Systems, 850 Lincoln Centre Drive Foster City, CA 94404 USA) using the following program: 1 cycle of 1 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 45 to 50°C and 45 s at 72°C, with a final extension of 7 min at 72°C, for SSR while the cycling condition for MAT primers was 1 cycle of 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 9 min at 72°C.

Amplified products were separated by electrophoresis (Bio-Rad, model 96, Bio-Rad Laboratories, Inc. Life Science Research Group 2000 Alfred Nobel Drive Hercules, CA 94547 USA) at 110 V for 2.0 h on 1.5% agarose gels and stained with intercalating ethidium bromide (0.5 µg ml^{-1}) using 1.0 × TBE buffer (89 mMTris, 89 mM borate, 2 mM EDTA pH 8.3). Gels were documented under UV-Trans-illuminator 2000 (Sagrate, Milan Italy). Isolates that were amplified with ITS primers, were submitted to Molecular and Cellular Imaging Center (MCIC), Wooster Ohio for Sanger sequencing.

Amplicons separated on a gel were scored using base pair (bp) size, in reference to the molecular marker for each genotype. Estimates of similarity among strains were calculated from the data matrices in the form of dissimilarity units and expressed as Euclidean genetic distance (Hintze, 1998). Polymorphic bands of SSR06 and SSR024 were subjected to power marker software for genetic variation calculation amongst the isolates.

Sanger sequenced products (forward and reverse) per isolate were assembled using MacVector software version 17.5.2 (Apex, NC, USA) to create a consensus sequence that was later blasted to the NCBI database for identification.

RESULTS

Selection of *E. turcicum* DNA by species specific primers

The rDNA ITS specific primers were detected in 20 out of

Table 1. Sequences of internal transcribed spacer ribosomal DNA (ITS) and mating type primers used for classification of *Exserohilum turcicum.*

Primer name	Sequence (5'… 3')	Gene type	Expected size (bp)	References
ITS1	TGTGTGTGTGTGTGTGTGTGT ATAAGACGGCCAACACCAAG	Specie specific	344	
MAT2-specific	ACCGATTGCTTCG CAAACATCTCAAGGCGGAA	Mat 1-2 & Mat2-2	195	Ramathan (2010)
MAT1 α-clone	GTGAACCGACCCTCAAC GTCCATGGGATACGCTACG	Mat 1-1 gene	190	

Source: Ramathan (2010).

Table 2. Simple sequence repeat primers used to characterize *Exserohilum turcicum* populations.

Primer name	Sequence	Accession No.	Reference
SSR01	5'-TAGTTGCAACCGAACAGG-3' 5'-CTCCGTAGGTATGATGGTGT-3'	AJ303015	Molina et al. (2001)
SSR06	5'-CGAACAGGACGAAAGAATAG-3' 5'-GTTTGTTCCAGTTCGTCAAG-3'	AJ303023	
SSR24	5'-TCAAGAGGAGAAGTTGA-3' 5'-GGTTCTGATCAAGAGGAGGA-3'	AJ303034	
SSR36	5'-ATTCCAGGTACGGCTACAC-3' 5'-ATTCAGATCTGGTCTGGTTG-3'	AJ303040	
SSR10	5'-GAGAGCATGAAAAGTGGAAA-3' 5'-CGTGACACTCGTCAGTTACA-3'	AJ303026	
SSR14	5'-ATTTGGTGAATGGGGTAAG-3' 5'-ACAGAGGGAAGCAAGTTTTT-3'	AJ303027	

Source: Molina et al. (2001).

38 isolates from maize samples, yielding the expected 344 base pairs (Figure 1). Fourteen isolates were Sanger sequenced and had 98 to 100% nucleotide identity with MN918438.1, MN918291.1, and query coverage of 94 to 100 with reference sequences in the NCBI Genbank. The sequenced isolates were annotated and deposited in the NCBI GenBank. The isolates, locations, altitude, and NCBI accession numbers are indicated in Table 3.

Genetic variation of *E. turcicum* based on SSR analysis

Molecular variations were observed among isolates based on microsatellite repeat makers used in this study (Figure 2). Two microsatellite regions, SSR 06 (Figure 2a) and SSR 024 (Figure 2b) were polymorphic, while SSR01, SSR10, SSR14 and SSR36 loci were monomorphic on the 14 *E. turcicum* isolates (Figure 2). A total of four alleles were identified in each locus (SSRs), and the genetic diversity ranged from 0.26 (SSR06) to 0.58 (SSR24). The analysis of the polymorphic bands using power marker software detected 4 and 5 genotypes for SSR 06 and SSR 024, respectively (Table 4).

The genotypes were distinguished by the type of polymorphic bands (Figure 2). The amplicons of isolates 2, 5 and 10 (Figure 2a) and 2, 5 and 6 (Figure 2b) were distinctly different genotypes, 4 and 8 are different from that of 9 and 13 genotypes while the other isolates genotypes were of the same strain, indicating five

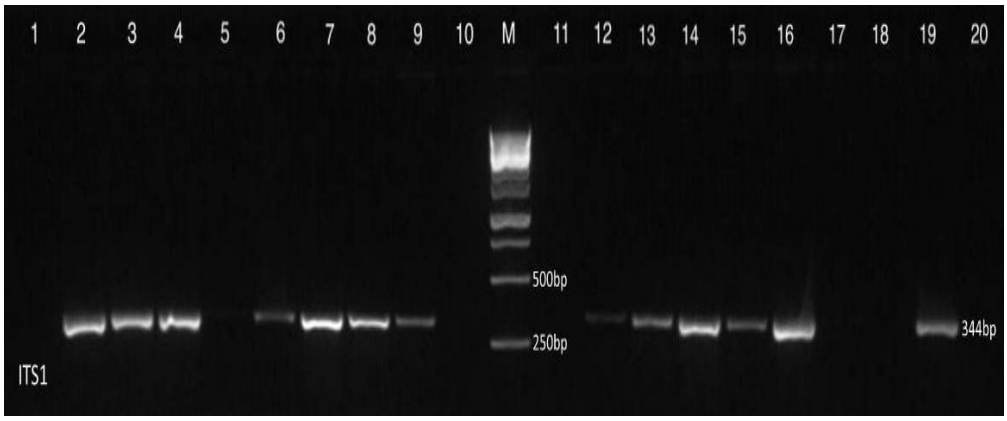

Figure 1. Gel image of rDNA ITS1 species specific primer showing 344 base pair PCR products specific to *E. turcicum* isolated from maize. Lanes 1-14 represents the sequenced isolates; MOR2, MOR3, IR4, MBY5, IR7, IR8, MOR9, MBY12, MBY13, IR14, MOR15, MOR16, IR19, IR20 in ascending order. Locations are described in Table 2, M = Marker
Source: Nwanosike, 2016.

Table 3. Tanzania strains of *Exserohilum turcicum* used in the study.

S/N	Region	Village	Co-ordinates	Altitude (masl)	*E. t* code	Sequenced DNA	MAT type
1	Mbeya	Mbaliu	8° 54' 11.53"S 33°26' 23.12"E	1777.1	MBY1	MT124702	NA
2	Morogoro	Msumbe	6° 53' 31.39"S 37°33' 41.98"E	566.1	MOR2	MT124699	2
3	Morogoro	Nanenane	6° 48' 05.24"S 37°40' 22.35"E	515.8	MOR3	MT124700	2
4	Iringa	Ifunda	8° 02' 51.08"S 35°28' 11.91"E	1869	IR4	MT124701	2
5	Mbeya	Uyole	8° 53' 46.55"S 33°32' 41.31"E	1913.2	MBY5	×	2
6	Mbeya	Igurusi	8° 49' 19.79"S 33°50' 33.09"E	1270.6	MBY6	NA	NA
7	Iringa	Mgama	8° 05' 56.56"S 36°30'51.61"E	365.2	IR7	MT124703	1
8	Iringa	Kalenga	7° 48' 13.40"S 35° 36' 04.14"E	1548.1	IR8	MT124704	2
9	Morogoro	Mazimbu	6° 46' 30.52"S 37°39' 31.27"E	506.1	MOR9	MT124705	2
10	Morogoro	Mikese	6° 43' 59.38"S 37°55' 12.63"E	393.9	MOR10	NA	NA
11	Njombe	Halal	3°25' 40.92"S 34°46' 00.00"E	1240.3	MBY11	NA	NA
12	Njombe	Makambako	8°50' 51.15"S 34°50' 50.36"E	1778.1	MBY12	MT124706	1
13	Mbeya	Iyunga	8°56' 13.90"S 33°25' 17.52"E	1733.9	MBY13	MT124707	1
14	Iringa	Ikengeza	7° 07' 36.40"S 35° 41' 00.00"E	1155.5	IR14	MT124708	2
15	Morogoro	Matombo	7° 07' 26.98"S 37° 48' 31.43"E	449.7	MOR15	MT124709	2
16	Morogoro	Bwakila	7° 57' 19.66"S 38° 12' 46.06"E	223.2	MOR16	MT124710	2
17	Iringa	Mfyome	7° 53' 56.38"S 35° 14' 04.05"E	1790.3	IR17	NA	NA
18	Iringa	Kibebe	7° 48' 10.00"S 35° 45' 23.08"E	1647.1	IR18	NA	NA
19	Iringa	Luganga	7° 14' 58.22"S 36° 06' 00.00"E	1249	IR19	MT124711	1
20	Iringa	Kitayawa	8° 01' 52.10"S 36° 11'58.39"E	987.1	IR20	MT124712	2

E. t = *Exserohilum turcicum*, x = PCR positive DNA isolates, masl = meters above sea level, MBY=Mbeya, IR=Iringa, MOR=Morogoro, NA = not applicable representing the strains with low quality DNA, 1 represents mating type 1, 2 represents mating type 2.
Source: Nwanosike, 2016.

genotypes of the pathogen. Polymorphic information content of SSR06 and SSR 024 loci were 0.25 and 0.52, respectively, with mean of 0.39 (Table 4). Although the two loci were genetically informative, SSR 024 indicated high heterozygosity (0.23) and gene diversity (0.58) compared to SSR 06.

Genetic variability of *E. turcicum* based on mating type genes

Gel electrophoresis identified both *MAT 1* and *MAT 2* (Figure 3). Amplification of isolates that were *MAT 1* (α-clone locus) yielded a fragment size of 190 bp (Figure 3a)

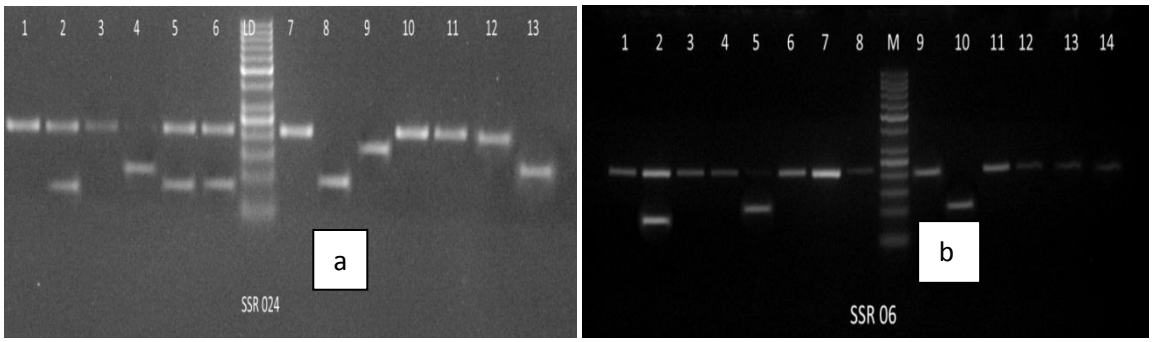

Figure 2. The polymerase chain reaction amplicons of maize derived *Exserohilum turcicum* strains generated by SSRs primer in a 1.5% agarose gel. Samples 1 - 14 and M/L DNA 100 base pair ladder, a = SSR 06 primer, b = SSR 024 primer.
Source: Nwanosike, 2016.

Table 4. Primer description, amplification, alleles, diversity, heterozygosity and polymorphic content of two SSR pairs, amplified polymorphic regions on 14 maize strains of *E. turcicum*.

Locus	Allele frequency	Genotype No.	Sample Size	No. of obs.	N_A	Availability	H	Heterozygosity	PIC
SSR 24	0.58	5	14	13	4	0.93	0.58	0.23	0.52
SSR 06	0.86	4	14	14	4	1.00	0.26	0.14	0.25
Mean	0.72	4.5	14	13.5	4	0.96	0.42	0.18	0.39

SSR = Simple sequence repeats, N_A = number of alleles, H = gene diversity, PIC = polymorphic information contents.
Source: Nwanosike, 2016.

while isolates for *MAT 2* (*MAT 2*- specific locus) produced fragment size of 195 bp (Figure 3b). Of the 14 isolates, four *MAT 1* (MBY 5, IR 8, IR 19 and MBY 13) and 10 *MAT 2* idiomorphs were observed. All Morogoro samples were *MAT 2* gene, while *MAT 1* and *2* existed in Mbeya, Njombe, and Iringa regions, however in an unequal proportion.

DISCUSSION

Information from the sequenced DNA and mating types indicated evidence of gene flow between populations of *E. turcicum* in the different maize fields of Tanzania. The microsatellites SSR06 and SSR24 loci and MAT type markers (*MAT1* and *MAT2*) polymorphism revealed strains of *E. turcicum*. The microsatellite regions of SSR06 and SSR24 loci fragment analysis of the 14 isolates revealed 4 allele. Genetic diversity of 0.42 and polymorphic information content of 0.39 (Table 4) demonstrated that sexual reproduction may have been responsible for the genotypes. Such results showed that five different strains of *E. turcicum* existed in the population. In South Africa, Haasbrock et al. (2014) reported 90 alleles across 13 SSR loci and gene diversity of 0.074 to 0.929 per locus, with average diversity of 0.602 in maize and sorghum *E. turcicum*. The study also showed minimum detection of 2 and maximum of 19 allele with amplification length of 191 to 493 bp, respectively.

The existence *MAT 2* dominated *MAT 1* in *E. turcicum* strains in an un-equal proportion, may be due to low frequency of genetic recombination, affected by location and genetic variation. The observed clones and mutants of *E. turcicum* isolated from Mbeya region could be due to frequent contact of sexually compatible strains, rapid and abundant inoculum and production of multiple generations and cycles of asexual reproduction. This may lead to high prevalence of disease in the Southern regions of Tanzania (Nwanosike et al., 2017). Maize is widely cultivated in Tanzania, particularly in the humid Southern Highlands, thus, the different climatic zones and cropping patterns as well as systems employed in different maize growing regions of the country may have also subjected the pathogen to different agro-ecological environments resulting in different mating types and genetic diversity.

The frequency and distribution of *MAT 1* and *MAT 2* in humid southern highlands of Mbeya, Njombe and Iringa regions indicated a possibility of sexual reproduction and a good potential for sexual recombination than in the dry lowland of Morogoro Region (Table 3). Random

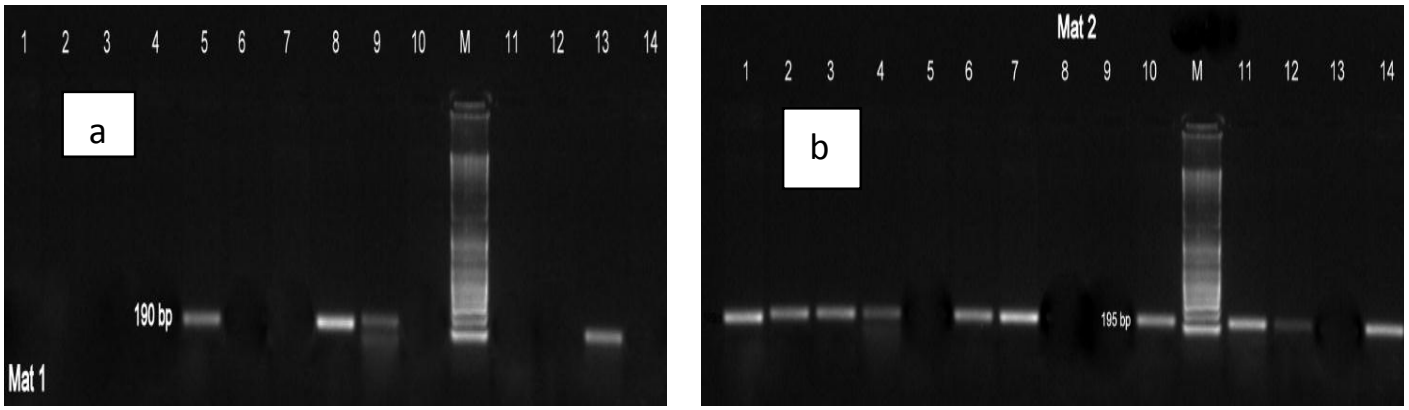

Figure 3a and b. PCR amplicons of *E. turcicum* strains a = α-clone gene (190 bp-*MAT 1*) and b = *MAT 2* specific gene (195 bp).
Source: Nwanosike, 2016.

distribution of mating types in the field may have enhanced chances of sexual reproduction (Welz et al., 1996). Borchardt et al. (1998b) also reported that severe epidemics of NLB in tropical environment resulted in higher population densities and more sexually compatible strains of *E. turcicum*. Similar condition was reported in Mbeya and Iringa regions, possibly due to consistent cultivation of susceptible maize cultivars, high *E. turcicum* population density and favourable climatic factors (Nwanosike et al., 2015).

Conclusion

The SSR and specific MAT markers used in this study explained variation in the genotypes of the 14 fungal isolates in Tanzania. The occurrence and distribution of *MAT 1* and *MAT2*, revealed the *E. turcicum* collected from Southern regions of Tanzania (Mbeya, Njombe, and Iringa) to be more diverse than isolates from Eastern regions (Morogoro). Following the current findings, there is a need for further diversity study on *E. turcicum* isolates collected from different zones of Tanzania by using robust molecular and sequencing techniques.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Association of serum MMP-9 with recurrence in patients with resectable esophageal squamous cell carcinoma after chemotherapy or concurrent radiotherapy

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Esophagectomy is a common standard treatment of localized disease. However, local recurrence frequently occurs in patients with advanced esophageal squamous cell carcinoma (ESCC) after operation, resulting in the need for using biomarkers to evaluate recurrence in patients with advanced ESCC during postoperative therapies. This study examined serum matrix metalloproteinase-9 (MMP-9) as a prognosis factor in recurrent patients with advanced ESCC after curative esophagectomy followed by chemotherapy or concurrent radiotherapy. During therapies, patients with recurrent tendencies always have low serum MMP-9 levels compared with those before treatment. For recurrent patients, a difference in recurrence-free survival rate is significant between MMP-9 ≥ 635 ng/mL and MMP-9 < 635 ng/mL before treatment (P < 0.05). Although serum MMP-9 is a negative prognostic factor for patients with recurrence tendency and cannot directly predict recurrence, low serum MMP-9 levels before treatment and after therapies still indicate high recurrence-free survival rate in patients with locally advanced resectable ESCC after chemotherapy or concurrent radiotherapy.

Key words: Matrix metalloproteinase-9, esophageal squamous cell carcinoma, recurrence, chemotherapy, survival.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a common histological subtype of esophageal cancer that occurs primarily in Eastern Asia and Eastern and Southern Africa (Torre et al., 2015). Chemotherapy or chemoradiotherapy is recommended as an adjuvant therapy following surgery due to metastasis and recurrence frequently occurring in patients with advanced ESCC (Lordick et al., 2016). The prognoses and times to death are similar in patients with node-negative and node-positive superficial ESCC once recurrence occurs (Ozawa et al., 2016). Therefore, patients with ESCC and high risk of recurrence after esophagectomy should receive additional chemoradiotherapy (Xue et al., 2018). Although advance therapies have improved the survival

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rate, the local recurrence remains high, leading to low cure rate (Brooks-Brunn, 2000). The late emergence of symptoms and the insensitivity and nonspecificity of biomarkers become challenges for prognostic prediction in patients with advanced ESCC (Siegel et al., 2017). Therefore, new sensitive biomarkers for ESCC prognostic prediction are urgent in patients with advanced ESCC after treatments.

Matrix metalloproteinase-9 (MMP-9) is a class of enzymes related to cancer pathogenesis because of its involvement in the degradation of extracellular matrix (Peisker et al., 2017). MMP-9 participates in tumorigenesis, and its high expression indicates poor prognosis in ESCC (Li et al., 2013; Gu et al., 2005). Serum MMP-9 is an effective biomarker in the diagnosis and prognosis of ESCC (Łukaszewicz-Zajac et al., 2009; Mroczko et al., 2008). Esophagectomy is the most common standard treatment for localized ESCC. However, recurrence rate remains high (Alidina et al., 2004). Although surgical resection is a potential mainstay for curable ESCC, locoregional recurrence is up to 23.8 to 58.0% of cases (Miyata et al., 2011; Lu et al., 2013; Guo et al., 2014). Therefore, the correlation of serum MMP-9 with recurrence in patients with locally advanced resectable ESCC should be determined.

This study investigates the prognosis of serum MMP-9 in recurrent patients with advanced ESCC after curative esophagectomy followed by chemotherapy or concurrent radiotherapy and evaluates the potential of serum MMP-9 as a recurrence predictor in patients with locally advanced resectable ESCC.

MATERIALS AND METHODS

Patients

From January 2012 to June 2016, 173 patients (stages III and IV) with advanced ESCC confirmed by histopathology and who underwent R0 resection were enrolled at Jiangsu Cancer Hospital (Nanjing, China). This study was performed in accordance with the Declaration of Helsinki and approved by the Biomedical Research Ethics Committee of Jiangsu Cancer Hospital. All participants provided informed consent.

Treatment modality

At the beginning, patients did not receive any treatment (before treatment). Then, all patients received chemotherapy at four different intervals, with about 1 to 2 months interval. A total of 57 patients received concurrent radiotherapy at the first course of chemotherapy with radiotherapy schemes, such as GTV60-65Gy/28-33f, CTV50-55Gy/28-33f and PTV50-66Gy/28-33f (after therapies). Chemotherapy was consistent with the previous description and included taxane combined with platinum, 5 fluorouracil and its derivatives combined with platinum, and gemcitabine combined with platinum (Ye et al., 2020). Five months after the end of chemotherapy, 57 patients had recurrence at the original lesion or metastasis, including lymph node and distal metastases, and classified as recurrent patients (recurrence patient group). Others were classified as nonrecurrent patients (nonrecurrence patient group). The recurrence patient group received further treatments, including chemotherapy or concurrent radiotherapy, again.

Serum samples and serum MMP-9 detection

Blood samples before and after chemotherapies (that is, the 1st, 2nd, 3rd and 4th cycles of treatment) were collected. The samples of recurrence patients were still collected at recurrence (re-0 cycle) and at course of further treatment (re-1 and re-2 cycle). Samples were stored at -80°C after centrifugation.

Human cytokine/chemokine panel (that is, MMP-9) was purchased from Millipore (CAT no. HMMP2MAG-55K-01; Millipore, USA) and performed in accordance with the manufacturer's instructions. Serum MMP-9 level was determined using the Luminex FLEXMAP 3D instruments and software supplied by Luminex Corporation (Austin, USA). The preparation of blood samples, setting of detection parameters, and calculation of serum MMP-9 concentrations were conducted in accordance with a previous study (Ma et al., 2017).

Statistical analysis

The concentrations of MMP-9 in serum were presented as mean ± SD. $P < 0.05$ indicated significance. Differences between two groups were analyzed using unpaired t-test. Comparisons among three or more groups were performed by ANOVA followed by pairwise comparisons by using the Bonferroni post hoc test. The follow-up ended on April 25, 2020, and the overall survival (OS) was calculated. Recurrence-free survival rates were calculated from the date of operation to the date of recurrence via the Kaplan-Meier method, and the significance of comparisons between groups was measured through the log-rank test. Cutoff values for OS and recurrence-free survival of serum MMP-9 were assessed by the receiver operating characteristic (ROC). The area under curve (AUC), sensitivity, and specificity were calculated. Statistical analyses were performed using a commercially available statistical software (that is, GraphPad Prism 5).

RESULTS

Serum MMP-9 decreased in recurrent and nonrecurrent patients with locally advanced resectable ESCC after therapies

The clinical characteristics of 173 patients with locally advanced resectable ESCC are detailed in Table 1. Serum MMP-9 levels were approximately equal between recurrent and nonrecurrent patients before treatment ($P > 0.05$, Figure 1A). However, serum MMP-9 levels significantly decreased in 116 nonrecurrent and 57 recurrent patients with locally advanced resectable ESCC after therapies compared with those before treatment ($P < 0.001$, Figure 1B). Furthermore, 57 recurrent patients maintained lower serum MMP-9 levels at recurrence than those before treatment ($P < 0.001$, Figure 1C). Serum MMP-9 levels did not increase until the third cycle of treatment in recurrent and nonrecurrent patients with locally advanced resectable ESCC after therapies (Figure 2A). Compared with that at recurrence, serum MMP-9

Table 1. Relation of serum MMP-9 before treatment to clinicopathological characteristics of 173 patients with locally advanced resectable ESCC (concentration unit: ng/mL).

Items		N	MMP-9		*P* value
			Mean*	SD	
Gender	Male	147	1064.09	1021.72	0.970
	Female	26	1071.96	698.35	
Age (years)	≤60	88	1050.51	706.47	0.979
	60-70	62	1077.69	1350.25	
	≥70	23	1088.26	671.75	
Stage	III	62	1048.52	749.53	0.867
	IV	111	1074.63	1088.40	
Recurrence	Yes	57	1046.93	789.63	0.863
	No	116	1074.28	1061.82	
Metastasis	Yes	144	1128.88	1046.76	0.056
	No	29	749.45	398.29	

*The concentration of serum MMP-9 at the pre-treatment.
Source: Author

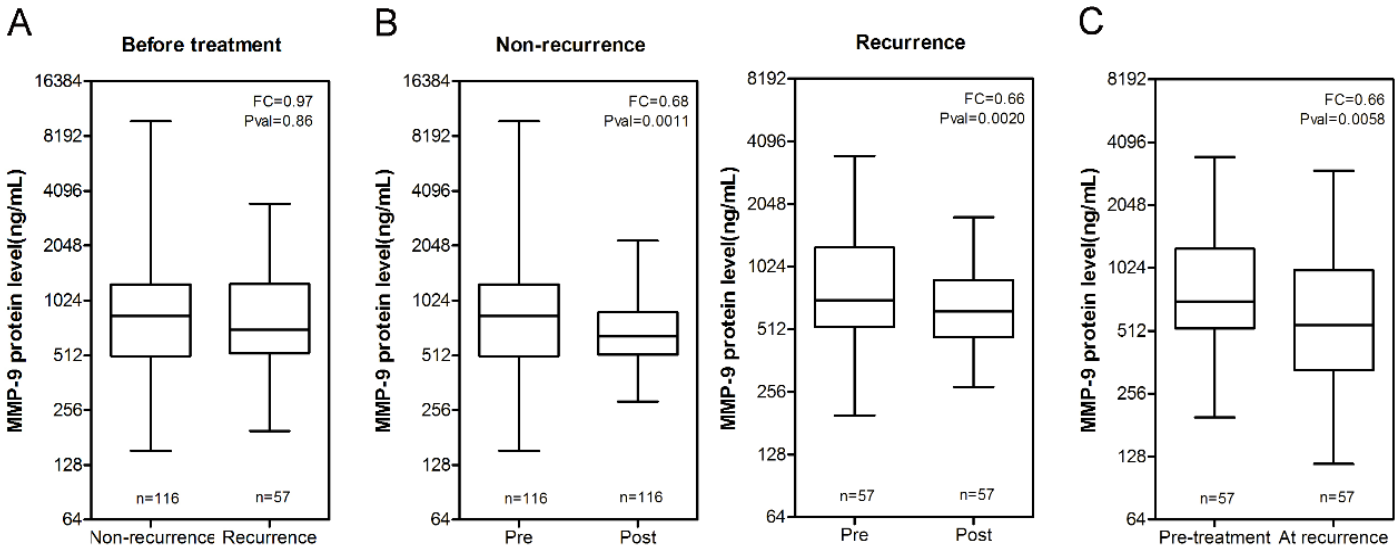

Figure 1. Boxplots of serum MMP-9 in patients with locally advanced resectable ESCC. (A) Comparison of serum MMP-9 between recurrent and nonrecurrent patients before treatment. (B) Comparison of serum MMP-9 before treatment and after therapies in nonrecurrent and recurrent patients respectively. (C) Comparison of serum MMP-9 before treatment and at recurrence in recurrent patients. Pre: Before treatment; Post: After therapies; FC: Fold change.
Source: Author

levels no longer decreased in recurrent patients during further treatments (P > 0.001, Figure 2B). No difference in serum MMP-9 level after therapies was observed between recurrent patients treated with chemotherapy and concurrent radiotherapy. However, nonrecurrent patients treated with concurrent radiotherapy had low serum MMP-9 than those treated with chemotherapy (P < 0.05, Figure 2C). Nonrecurrent and recurrent patients treated with concurrent radiotherapy at the 2nd, 3rd, and 4th cycles of treatments had significantly decreased

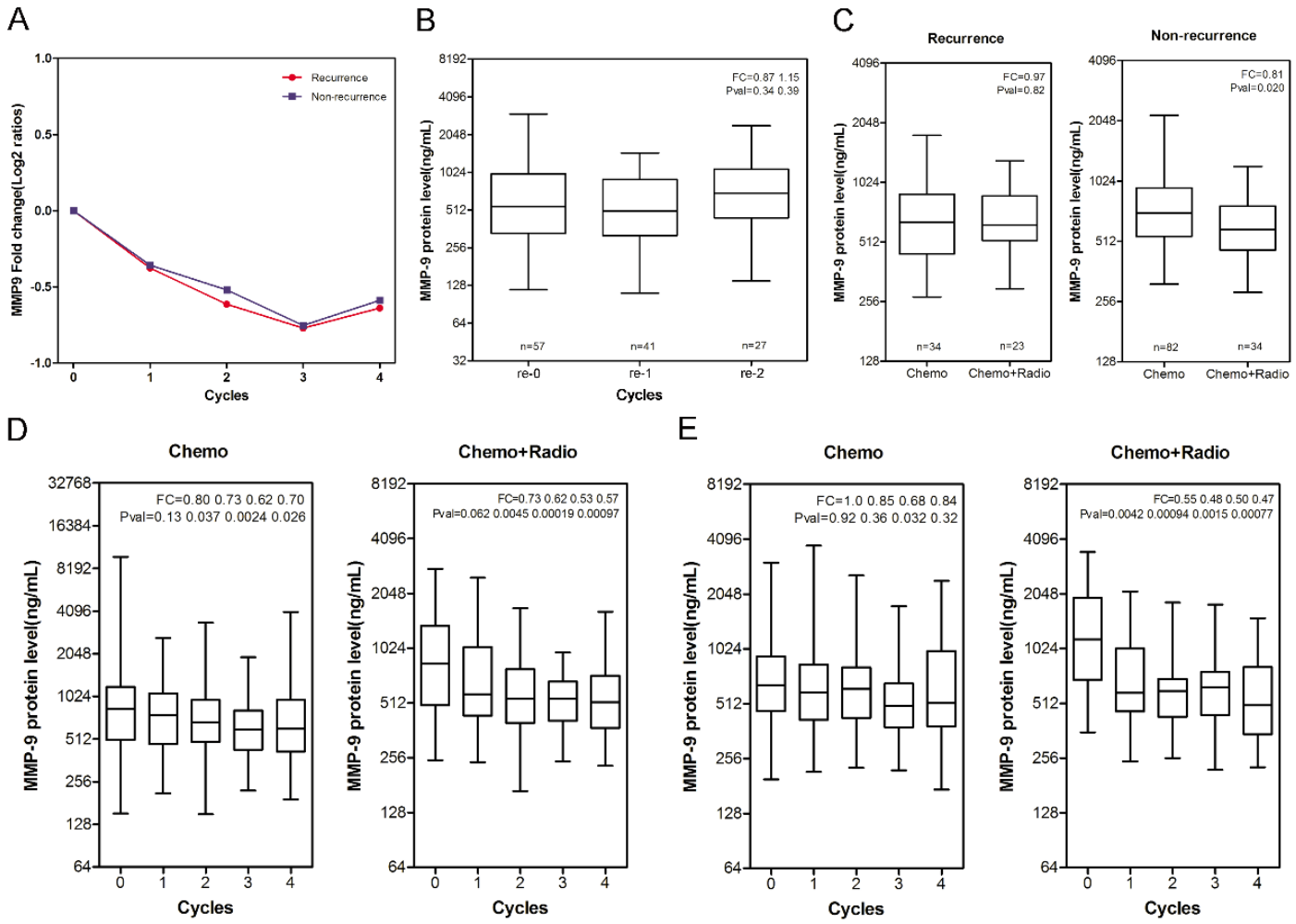

Figure 2. The changes of serum MMP-9 level in locally advanced resectable ESCC patients during chemotherapy or concurrent radiotherapy. (A) Mean change of serum MMP-9 in recurrent and nonrecurrent patients. (B) Boxplots of MMP-9 in 57 recurrent patients after further treatment. (C) Comparison of serum MMP-9 between chemotherapy and concurrent radiotherapy in recurrent and nonrecurrent patients respectively. The median value of MMP-9 concentration from pre-treatment (0 cycle) to the first, second, third and fourth cycles of treatment (1, 2, 3 and 4 cycles) in (D) nonrecurrent patients and (E) recurrent patients treated with chemotherapy or concurrent radiotherapy, respectively. Chemo: Patients received at least four times of chemotherapy. Chemo+Radio: Patients received concurrent radiotherapy at the first cycle of chemotherapy. FC: Fold change. Re-0: At recurrence; re-1: The first cycle of further treatment after recurrence; re-2: The second cycle of further treatment after recurrence.
Source: Author

serum MMP-9 levels compared with those before treatment ($P < 0.001$, Figures 2D and 2E). Nonrecurrent patients treated with chemotherapy had significantly lower serum MMP-9 levels at the 2nd, 3rd and 4th cycles of treatments than those before treatment ($P < 0.001$), whereas the opposite was observed in recurrent patients ($P > 0.05$, Figures 2D and E).

Only non-recurrent patients with low serum MMP-9 had long survival time

The OS values of recurrent and non-recurrent patients were approximately equal ($P > 0.05$, Figure 3A). However, for recurrent patients, serum MMP-9 ≥ 980 ng/mL at recurrence, MMP-9 ≥ 521 ng/mL after treatment, and MMP-9 ≥ 521 ng/mL after further treatment were not independent factors for OS ($P > 0.05$; Figure 3B to D). Furthermore, in recurrent patients with locally advanced resectable ESCC, concurrent radiotherapy did not prolong OS compared with chemotherapy ($P > 0.05$, Figure 4A). Recurrent patients with serum MMP-9 < 635 ng/mL before treatment and MMP-9 < 521 ng/mL after therapies had long OS. However, serum MMP-9 ≥ 635 ng/mL before treatment and ≥ 521 ng/mL after therapies were still not determined as independent factors for OS

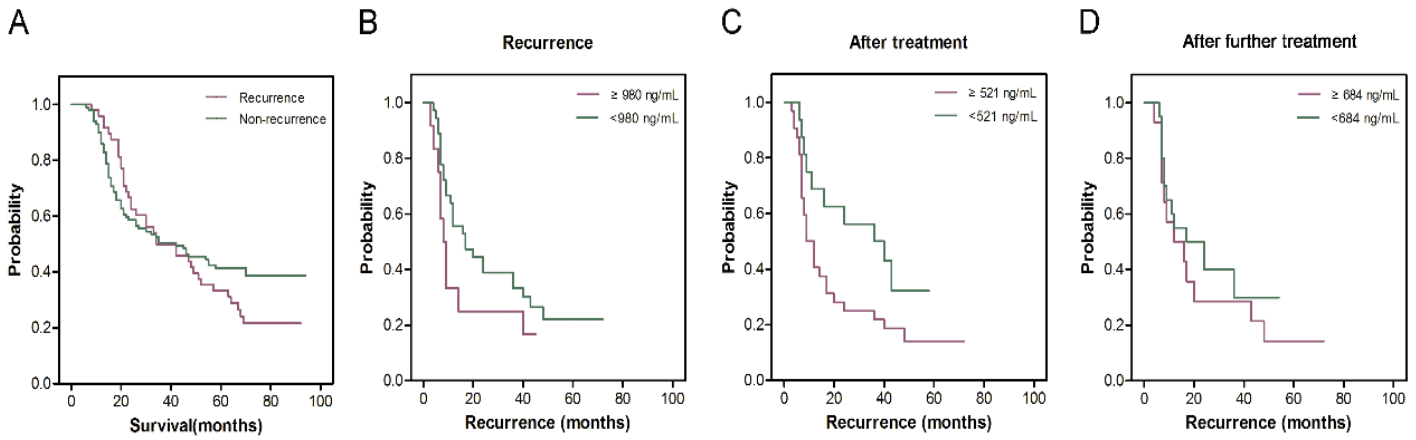

Figure 3. The prognosis values of serum MMP-9 in recurrent patients with locally advanced resectable ESCC after recurrence. (A) Survival with recurrent patients and nonrecurrent patients. Hazard ratio=1.222 (95% CI, 0.7976-1.871), P=0.3574. (B) Survival from recurrence to end of follow-up with high (≥ 980 ng/mL) and low (< 980 ng/mL) serum MMP-9 at recurrence. Hazard ratio=1.820 (95% CI, 0.7698-4.305), P=0.1725. (C) Survival from recurrence to end of follow-up with high (≥ 521 ng/mL) and low (< 521 ng/mL) serum MMP-9 after therapies. Hazard ratio=1.897 (95% CI, 0.9611-3.743), P=0.0649. (D) Survival from recurrence to end of follow-up with high (≥ 521 ng/mL) and low (< 521 ng/mL) serum MMP-9 after further treatment. Hazard ratio=1.360 (95% CI, 0.5942-3.114), P=0.4665.
Source: Author

(P > 0.05, Figures 4B and 4C). However, nonrecurrent patients treated with concurrent radiotherapy had significantly longer OS compared with patients treated with chemotherapy (P < 0.05, Figure 4D). Survival times in nonrecurrent patients with MMP-9 ≥ 475 ng/mL and MMP-9 < 475 ng/mL before treatment were not different (P > 0.05, Figure 4E). However, serum MMP-9 ≥ 700.25 ng/mL after therapies was an independent factor for OS in nonrecurrent patients with locally advanced resectable ESCC. Nonrecurrent patients with low serum MMP-9 (< 700.25 ng/mL) had significantly longer OS than those with high serum MMP-9 (≥ 700.25 ng/mL) after therapies (P < 0.001, Figure 4F).

Serum MMP-9 was associated with recurrence-free survival rate in patients with locally advanced resectable ESCC

Among 173 patients with locally advanced resectable ESCC, patients with serum MMP-9 < 577 ng/mL had significantly higher recurrence-free survival rates than those with MMP-9 ≥ 577 ng/mL before treatment (P = 0.0411, Figure 5A). Furthermore, compared with those with MMP-9 ≥ 700.25 ng/mL after therapies, 173 patients with MMP-9 < 700.25 ng/mL had higher recurrence-free survival rates (P < 0.001, Figure 5B). For recurrent patients, the difference in recurrence-free survival rate was significant between MMP-9 ≥ 635 ng/mL and MMP-9 < 635 ng/mL before treatment (P = 0.0483, Figure 5C). However, no significant difference in recurrence-free survival rate was observed between 57 nonrecurrent patients with MMP-9 ≥ 521 ng/mL and MMP-9 < 521 ng/mL after therapies (P = 0.2613, Figure 5D).

DISCUSSION

Radical esophagectomy is regarded as a curative treatment for resectable ESCC in China (Feng et al., 2020). However, many patients develop locoregional recurrence and distant metastasis after surgery (Li et al., 2013; Shen et al., 2017). In this study, although all resectable ESCC patients maintained a low level of serum MMP-9 during therapies including chemotherapy or concurrent radiotherapy, some patients still develop recurrence. It suggests that serum MMP-9 after therapies was not a direct marker of recurrence in resectable ESCC patients treated with chemotherapy or concurrent radiotherapy. However, it does not mean that serum MMP-9 cannot be used as a prognostic factor for patients with resectable ESCC after therapies. After therapies, low serum MMP-9 is beneficial to prolong the survival time of resectable ESCC patients. This result is consistent with the report of Ye et al. (2020) who indicated that serum MMP-9 is a potential prognostic biomarker for response to chemotherapy or concurrent radiotherapy in patients with ESCC. Li et al. (2019) reported that the overexpression of MMP-9 predicts poor prognosis in Kazakh patients with ESCC. In addition, the level of serum MMP-9 before treatment is negatively correlated with the recurrence-free survival rate in patients with resectable ESCC after chemotherapy or concurrent radiotherapy, indicating that for patients with recurrent tendency, high level of serum MMP-9 before treatment is easy to recurrence in a short time.

However, findings about serum MMP-9 in prognosis of esophageal cancer (EC) are controversial. MMP-9 is not a potential biomarker in the prognosis of EC (Mroczko et al., 2008). A meta-analysis indicated that the

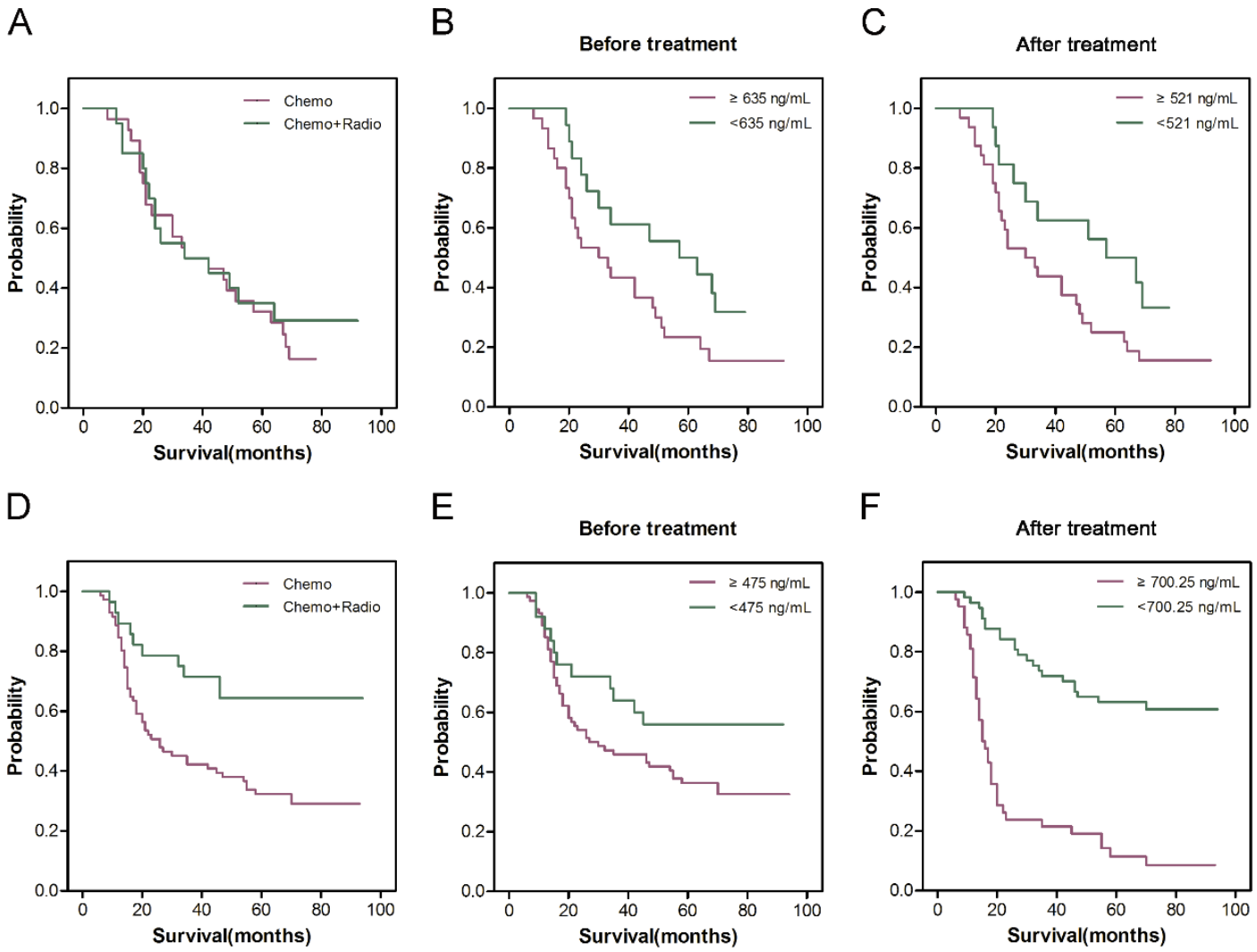

Figure 4. Overall survival from diagnosis to end of follow-up with serum MMP-9 in recurrent and nonrecurrent patients. (A) Overall survival with chemo group and chemo+radio group in recurrent patients. Hazard ratio=1.197 (95% CI, 0.6186-2.314), P=0.5940. (B) Overall survival with high (≥ 635 ng/mL) and low (< 635 ng/mL) serum MMP-9 before treatment in recurrent patients. Hazard ratio=1.845 (95% CI, 0.9559-3.561), P=0.0679. (C) Overall survival with high (≥ 521 ng/mL) and low (< 521 ng/mL) serum MMP-9 after therapies in recurrent patients. Hazard ratio=1.877 (95% CI, 0.9669-3.644), P=0.0628. (D) Overall survival with Chemo group and Chemo+Radio group in nonrecurrent patients. Hazard ratio=2.228 (95% CI, 1.296-3.831), P=0.0038. (E) Overall survival with high (≥ 475 ng/mL) and low (< 475 ng/mL) serum MMP-9 before treatment in nonrecurrent patients. Hazard ratio=1.681 (95% CI, 0.9535-2.965), P=0.0726. (F) Overall survival with high (≥ 700.25 ng/mL) and low (< 700.25 ng/mL) serum MMP-9 after therapies in nonrecurrent patients. Hazard ratio=6.868 (95% CI, 3.821-12.35), P < 0.0001.
Source: Author

overexpression of MMP-9 is a potential independent prognosis factor of patients with ESCC in Asia (Zeng et al., 2013). This finding may be because the significance of MMP-9 as a prognostic factor is disaccorded by different patient populations as research subjects. In the present study, these recurrent patients maintain low serum MMP-9 levels during the progress of therapies until recurrence. Although serum MMP-9 < 521 ng/mL after treatment cannot prolong the survival of recurrent patients, serum MMP-9 no longer decreases in recurrent patients who have undergone further treatment after recurrence. The survival time from recurrence to the end of follow-up is not related to serum MMP-9 level at recurrence and after further treatment in recurrent patients with locally advanced resectable ESCC. Low serum MMP-9 before treatment and after therapies cannot prolong the survival time of recurrent patients with locally advanced resectable ESCC, suggesting that serum MMP-9 is a negative prognostic factor for patients with recurrence tendency. However, high MMP-9 levels (≥ 700.25 ng/mL) after therapies are associated with poor prognosis in nonrecurrent patients with locally advanced

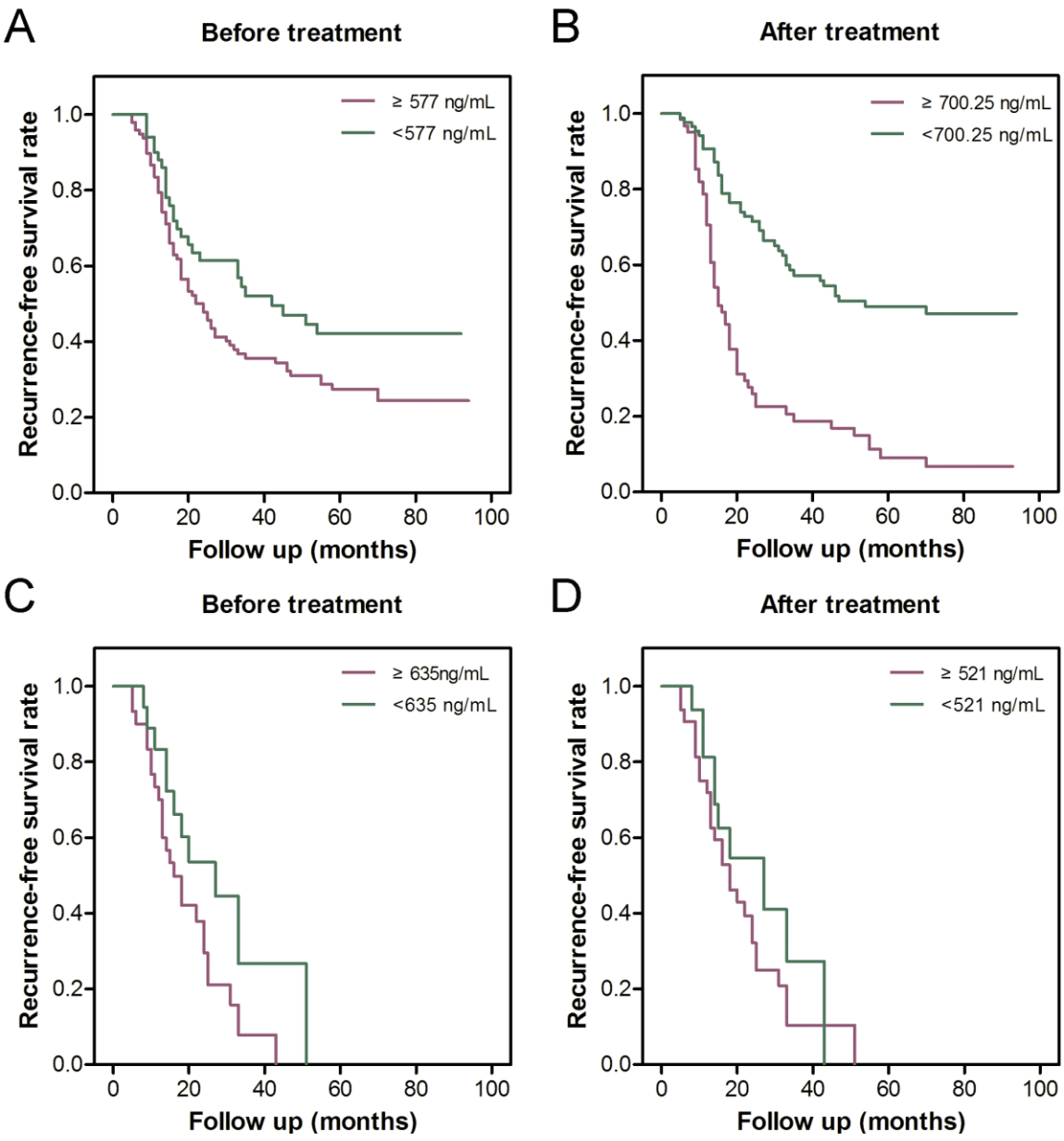

Figure 5. Kaplan-Meier analysis of recurrence-free survival rates. Comparison between high and low serum MMP-9 (A) before treatment, hazard ratio=1.544 (95% CI, 1.018-2.343), P=0.0411; (B) after therapies, Hazard ratio= 4.118 (95% CI, 2.621-6.469), P < 0.0001 in 173 patients. Comparison between high and low serum MMP-9 (C) before treatment, hazard ratio=1.997 (95% CI, 1.005-3.970), P=0.0484; (D) after therapies, hazard ratio=1.497 (95% CI, 0.7405-3.026), P =0.2613 in 57 recurrent patients.
Source: Author

resectable ESCC.

Esophagectomy is a common standard for the treatment of localized disease. However, local recurrence rate is high in patients with EC after operation (Alidina et al., 2004). Neoadjuvant radiochemotherapy improves the 5-year survival rate in patients with EC (van Hagen et al., 2012; Shapiro et al., 2015). Neoadjuvant chemoradiotherapy followed by surgery prolongs survival time over surgery alone in patients with locally advanced ESCC (Yang et al., 2018). In the present study, 173 patients with advanced ESCC have undergone esophagectomy followed by chemotherapy or concurrent radiotherapy. A total of 57 recurrent patients after cycles of chemotherapy or concurrent radiotherapy are observed. Postoperative nonrecurrent patients who received concurrent radiotherapy have longer survival time than those treated with chemotherapy alone indicating that locally advanced resectable ESCC patients

without recurrence obtains benefits from concurrent radiotherapy. Nonrecurrent patients treated with concurrent radiotherapy have maintained lower serum MMP-9 levels than those treated with chemotherapy alone during the progress of treatments. However, serum MMP-9 levels are not significantly low in recurrent patients treated with concurrent radiotherapy compared with those treated with chemotherapy. This finding means that serum MMP-9 is related to survival time and can be used as a potential prognostic factor. Without regard for recurrence, concurrent radiotherapy is a suitable therapeutic regimen for patients with locally advanced resectable ESCC.

This study has limitations. This study is a retrospective analysis based on existing data rather than selecting specific recurrent patients into the group. Therefore, few other risk factors are analyzed when predicting recurrence through serum MMP-9 in patients with locally advanced resectable ESCC. In addition, patients with recurrence at the original lesion or metastasis are classified as recurrence patient group in this study rather than patients with ESCC and local recurrence merely after curative esophagectomy followed by chemotherapy or concurrent radiotherapy.

Conclusion

Although serum MMP-9 after therapies is not a direct marker of recurrence, serum MMP-9 level before treatment is negatively associated with recurrence-free survival in locally advanced resectable ESCC patients, suggesting that serum MMP-9 before treatment is a potential risk factor for recurrence in ESCC patients after curative esophagectomy followed by chemotherapy or concurrent radiotherapy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Rhizosphere competence and abiotic stress tolerance of commercially sold *Pseudomonas fluorescens* biofertilizers: Implications for their bioremediation potential

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Microbial remediation research has become front and centre gaining momentum for numerous environmental biotechnological applications. Agronomical bioproducts have been widely used for many years and their availability, low costs and safety makes them potentially suitable for alternative biotechnological applications, specifically environmental remediation. In this study, the commercially available biofertilizer, *Pseudomonas fluorescens* (Rizofos), was examined for its rhizosphere competence, abiotic stress tolerance and heavy metal tolerance using relatively rapid and economically feasible standard culture-based laboratory methods to determine their potential use in the field of bioremediation. This plant growth promoting bacteria biofertilizer shows phase variation, metabolic versatility, and mobility, all of which are necessary for rhizosphere fitness and colonisation. In addition, both exceptional abiotic stress tolerance (pH, NaCl and temperature) and heavy metal (HM) tolerance was exhibited by this biofertilizer. To conclude, this study demonstrates that the prospect of using already available, well studied, safe and environmentally friendly agronomic bioproducts as alternative biotechnologies including bioremediation is realistic offering a more rapid solution to environmental problems extending beyond identifying, testing and formulating new liquid bioinoculants specifically for contaminated soils.

Key words: Agricultural, bioproducts, *Pseudomonas fluorescens*, bioremediation.

INTRODUCTION

The economic growth of South Africa is primarily fuelled by land use activities, mining and non-renewable resource exploitation. However, the environmental impacts associated to these activities are extensive both in scale and severity. Natural resource utilization driven economic growth in South Africa urgently needs to

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become uncoupled from environmental degradation for sustainable development (Soderholm, 2020). Biotechnology offers a solution to overcoming the challenges associated with sustainable development in South Africa. However, biotechnology development and its integration into policy and management must be conceptualized and emerge upon the foundation of practical applicability in a developing-country context. Innovation and technology transfer offer such an avenue to allow the introduction, advent, and expansion of environmental biotechnology into South Africa.

The use of biofertilizers for sustainable agriculture represents one of the most advanced biotechnological tools in modern times. Across the world, at an annual increase of ~10%, there is an expanding market for microbial inoculants (Berg, 2009). When compared to chemical pesticides and fertilisers, microbial inoculants offer several advantages: They are safer with more targeted activity and reduced environmental damage. They are also effective in smaller quantities with the ability to multiply, and have decomposition procedures that are quicker (Berg, 2009).

The legacy of environmental pollution has followed in the wake of worldwide industrialisation and extensive agricultural and anthropogenic practices (Shinwari et al., 2015). Toxic compounds, metals and effluents have heavily contaminated soils, water and air leaving an environment that is unsuitable for sustaining life. Conventional methods for the remediation of contaminated soils and mine sites are high energy consuming, costly and in large unsustainable (Jeyasingh and Philip, 2005). In this regard, microbial based technologies, known as bioremediation, to remediate or assist remediation practices have gained considerable attention over the last few decades due to their superior performance, low cost, and environmentally friendly nature (Iqbal and Edyvean, 2004; Wu et al., 2006). Bioremediation is defined as the implementation of biological systems, almost universal microorganisms, to clean up or restore contaminated sites (Kumar et al., 2018).

The *Pseudomonas* genus is one of the best-studied and most important bacterial taxa in soil (Raaijmakers et al., 2002). The reasons for this are that the *Pseudomonas* species are isolated easily from the natural environment, effortless to cultivate and easy to manipulate genetically (Whipps, 2001; Raaijmakers et al., 2002). The members of the genus can colonise a wide range of niches as they demonstrate wide metabolic diversity (Palleroni and Cornelis, 2008). Other important characteristics of the genus *Pseudomonas* include rapid growth, production of metabolites (siderophores and growth promoters) and their ability to adapt to environmental stress and compete with other microorganisms (Goldberg, 2000; Kraemer, 2004; Hider and Kong, 2010; Cornelis, 2010; David et al., 2018). These features combine to make this microbial inoculant a strongly desired agent in applications such as bioremediation and biocontrol (Kloepper et al., 2004; Weller, 2007). *Pseudomonas fluorescens* is one species of this diverse group of bacteria that is particularly well known for its role in biocontrol, biodegradation, and bioremediation (Roca and Olsson, 2001; Palleroni, 2010).

The environmental biotechnological applicability of certain microbial species is generally performed by isolating soil microbes from the contaminated site and testing whether they possess the capacity to accomplish the desired function. This technique is however, limited by being site-specific and if the microbe presents as being applicable for bioremediation further time, money and workforce are required to developing the bacteria into a formulated inoculant that is safe for both the environment and human handling. This process is in all regard paradigmatic in the developing world where environmental conciseness and funds are not as broadly or extensively prioritized. Considering this, the use of commercially available, safe and relatively cheap PGPB biofertilizers exhibiting multiple plant beneficial properties, abiotic stress and metal tolerance is an encouraging, environmentally friendly and cost competitive soil bioremediating tool. The prospect of using commercially available biofertilizers for bioremediation technology and establishing whether the administration of PGPR biofertilizers, largely used to improve agricultural yields, for solving environmental problems is a concept that has in large been overlooked (Figure 1).

P. fluorescens containing biofertilizers produced specifically for their phosphate solubilising properties are a commercially available agricultural biotechnology (Goddard et al., 2001). Compared to fungi, the faster growth rate and ease for manipulation of bacteria make them convenient to culture. Thus, may serve potentially as more suitable effectors for the bioremediation of soil. Being commercially available and widely used, its performance for scale-up and field employment has already been confirmed and is a significant factor to why the commission of bioinoculants developed for biotechnological employment are commonly short-lived (Goddard et al., 2001). Numerous in-depth studies have exposed the bioremediation potentials of *P. fluorescens* (Juhasz and Naidu, 2000; Barathi and Vasudevan, 2001; Janek et al., 2010) and therefore this species was selected for this research. Thus, the microbial biofertilizer was investigated for its potential to be used beyond its current agricultural purpose in the realm of bioremediation and bioaugmentation-assisted rehabilitation of soils.

The aim of this work is to determine whether this biofertilizer shows morphological, behavioral, and metabolic phenotypes pertaining to rhizosphere colonization ability and survival. The objectives of this study were to use a culture-based strategy to examine colony morphology expressions, abiotic stress tolerance and heavy metal tolerance of *P. fluorescens*. PGPR *P. fluorescens* was cultured on a variety of variable culture

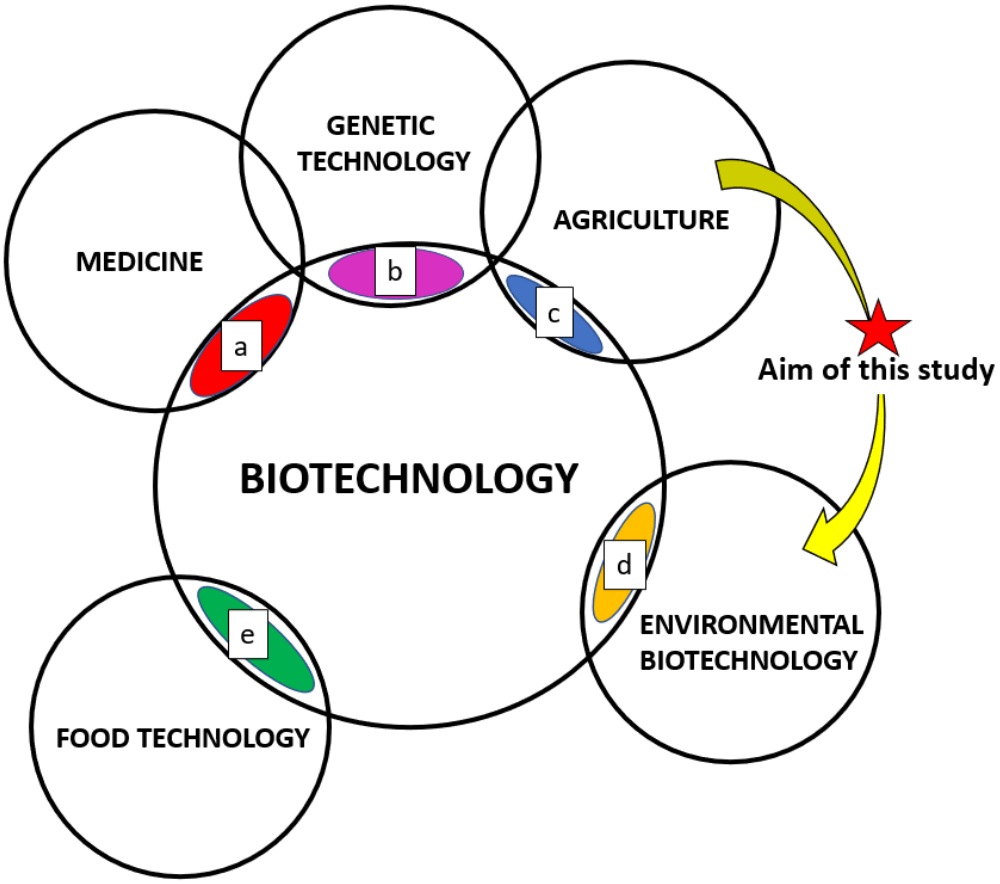

Figure 1. Applications of biotechnology and alternative applications of agricultural biotechnology in environmental biotechnology. (a) Antibiotic production; (b) Genetic engineering for human application; (c) Genetic engineering for plant and animal applications; (d) Decontamination, biomonitoring, biosensors and pollution prevention, and (e) Fermentation products (adapted from Gavrilescu, 2010).
Source: Author

media and colony growth times and the observed phenotypic expressions (colony morphology, alterations in colony traits, growth rate, fluorescent pigments and colouration) were examined and compared. Abiotic stress and heavy metal tolerance was also studied using standard culturing techniques and determined by adjusting the individual environmental parameters for each and inspecting the effects on growth. Identifying the impacts of some variables on the microbiological effects and phenotypic expressions of *P. fluorescens* provides inferences on their potential environmental adaptability, virulence and resistance following introduction into the soil environment (Sousa et al., 2013). As this microbe is already used in agriculture for its plant growth promoting abilities, these properties were not studied as the goal of this study was to determine if the use of this agricultural bioproduct in environmental biotechnological applications is a feasible recourse which mainly relying on its ability to survive, replicate, tolerate and perform the desired biotechnological appointment.

MATERIALS AND METHODS

Microorganism, maintenance and storage

The bacterial strain of *P. fluorescens* used in this study was donated as a commercially sold inoculant labelled Rizofos maize from Microbial Biological Fertilizers International (MBFi), Delmas, South Africa. Rizofos maize is a liquid inoculant containing the phosphorus solubilizing bacteria *(Pseudomonas fluorescens)* (1 × 10^9 cfu/mL in a sterile liquid broth culture). This strain is however also capable of producing siderophores under iron limited conditions a trait desired for this experiment. This biofertilizer is a special liquid formulation that contains not only the desired micro-organism and their nutrients, but also special cell protectants or substances that encourage longer shelf life and tolerance to stressful condition. The *P. fluorescens* was maintained at 4°C on nutrient agar (Merck, Darmstadt, Germany) plates. For long time storage, stock cultures were made from liquid cultures (1200 µl culture, 300 µl 80% glycerol) and frozen at -20°C.

Several growth-based assays were used to phenotypically characterise the *P. fluorescens* biofertilizer. These assays are important to increase understanding of the ecology of this significant rhizosphere related microorganisms and have major implications for its application as a bioinoculant for environmental

Table 1. Media used and their composition.

Media	Composition
Pseudomonas agar	Tryptone (10 g/l) Gelatin peptone (16 g/l) Potassium sulphate (10 g/l) Magnesium chloride (anhydrous) (1.4 g/l) Agar (11 g/l)
Kings B agar	Peptone (20 g/l) Dipotassium hydrogen phosphate (1.5 g/l) Magnesium sulphate heptahydrate (3 g/l) Glycerol (10 ml/l) Agar (15 g/l)
LB agar	Sodium chloride (10 g/l) Tryptone (10 g/l) Yeast extract (5 g/l) Agar (15 g/l)
Nutrient agar	Beef extract (10 g/l) Peptone (10 g/l) Sodium chloride (5 g/l) Agar (15 g/l)

Source: Author

restoration and remediation biotechnologies.

Media composition and preparation of experimental plates

P. fluorescens was cultured in four different media, Pseudomonas agar, Kings B agar, LB agar and nutrient agar to investigate the effects of the composition of different media and growth time on (i) morphological features (that is, colony growth, colour and edge), (ii) rate of bacterial growth and (iii) physiological features (that is, colour changes and fluorescens). To assess the impact of solid media composition on colony morphology, growth and fluorescens, *P. fluorescens* was streaked on different solid media (Table 1).

The media were prepared, autoclaved at 121°C for 20 min, allowed to cool and poured into Petri dishes under sterile conditions in triplicate. As the amount of solid medium in the Petri plates has been observed to impact colony morphogenesis (Sousa et al., 2013), the height of the solid media in each plate was standardised to 0.5 cm thick or ~15 mL of medium per 9 cm diameter plates with an area of 7226.4 mm^2.

Detection of motility

Motility can be divided into (i) swimming and (ii) swarming forms (Robertson et al., 2013). A positive result for swimming motility is shown by a ring of colony expansion after 24 to 48 h of growth. Swarming motility is observed if the edge of isolated colonies demonstrates irregular extensions and/or projections after 24 to 48 h of growth. Colony morphogenesis and motility were monitored visually over the incubation period.

Detection of fluorescence

After 24, 48 and 72 h of incubation, the plates were examined for fluorescence under an ultraviolet light at 360 nm.

Abiotic stress tolerance

pH tolerance tests

To study the effect of temperature on growth of *P. fluorescens*, a loopful of 24-h old culture growth in nutrient broth were streaked onto nutrient agar plates with different pH values (4, 5, 6, 7, 8, 9 and 10). The final pH of the medium was adjusted using 1 mol/L HCl or 1 mol/L NaOH. The plates were incubated at 25°C for 48 h and visually inspected for the absence or presence of growth, which were then documented as intolerant (I) and tolerant (T), respectively.

Temperature tolerance tests

To study the effect of temperature on growth of *P. fluorescens*, a loopful of 24-h old culture growth in nutrient broth were streaked onto nutrient agar plates and incubated at different temperatures of 5, 10, 15, 20, 25, 30, 35 and 40°C for 48 to 96 h (Bruno et al., 2020). The plates were visually inspected for the absence or presence of growth which was then documented as intolerant (I) and tolerant (T), respectively.

Salinity tolerance tests

To study the effect of temperature on growth of *P. fluorescens*, a loopful of 24-h old culture growth in nutrient broth were streaked onto nutrient agar plates amended with different concentrations of NaCl (0.1, 1, 2, 3, 4, 5, 6 and 7%). The plates were incubated at 25°C for 48 h and visually inspected for the absence or presence of growth which was then documented as intolerant (I) and tolerant

Table 2. Heavy metal concentrations and volumes added to agar plates.

Concentration (ppm)	Iron (µM)	Manganese (µM)	Cobalt (µM)	Zinc (µM)	Copper (µM)	Lead (µM)	Chromium(µM)
ppm				µM			
10	38.46	66.22	42.03	73.37	40.05	30.193	100
50	192.3	331.1	210.15	366.84	200.25	150.97	500
100	384.6	662.2	420.3	733.7	400.5	301.93	1000
200	769.2	1324.5	840.6	1467.4	801	603.9	2000
300	1153.8	1986.7	1261	2201	1201.5	903.8	3000
ppm			**Volume (mL) to add to agar ($(C_1)(V_1) = (C_2)(V_2)$)**				
10	0.01	0.02	0.01	0.02	0.01	0.008	0.025
50	0.05	0.08	0.05	0.09	0.05	0.04	0.125
100	0.09	0.2	0.1	0.2	0.1	0.08	0.25
200	0.2	0.3	0.2	0.4	0.2	0.15	0.5
300	0.3	0.5	0.3	0.55	0.3	0.22	0.75

Source: Author

(T), respectively.

Heavy metal (HM) tolerance

The heavy metals used in the tolerance tests were iron, manganese, cobalt, zinc, copper, lead and chromium from $FeSO_4.6H_2O$, $MnSO_4.4H_2O$, $CoCl_2.6H_2O$, $ZnCl_2$, $CuO_4S.5H_2O$, $Pb(NO_3)_2$ and CrO_3, respectively. A 1 mol/L stock solution for each metal salt was made from which the appropriate volumes were taken to get the desired concentration of heavy metals in each of the 25 mL agar plates using $C_1V_1 = C_2V_2$ (Table 2).

The heavy metal (HM) tolerance of the PGPR *P. fluorescens* was tested using the agar dilution method (Lee et al., 2009). The *P. fluorescens* was grown in a nutrient broth for 24 h and then, using an inoculating loop, streaked onto nutrient agar plates amended individually with increasing concentrations (10, 50, 100, 200 and 300 ppm) of different heavy metals. Unamended nutrient agar plates were used as controls to examine tolerance. The plates were incubated at 25°C for 48 h and visually inspected for the absence or presence of growth which was then documented as intolerant (I) and tolerant (T), respectively.

RESULTS

Effect of medium composition on colony morphology, colour and growth

The PGPR *P. fluorescens* was plated onto different agar media to assess the effect of nutritional composition on colony morphology and biomass production. The time-lapse images of the plates at 24, 48 and 72 h are given for each medium in Figures 2 to 5.

Colony growth and expansion of the *P. fluorescens* strain over the course of 72 h showed variations in rate and extent amongst the four media types. Colony growth, degree of expansion and rate of expansion was greatest on the Kings B agar medium and slowest on the nutrient agar medium.

Motility was not observed on the nutrient agar medium. Swimming motility was observed on the Pseudomonas agar medium. The Kings B agar media was dominated by swarming motility growth. The LB agar medium showed both swimming and swarming motility and growth behaviours. Unlike the thick irregular tendrils observed on the Kings B agar, those on the LB agar plates where longer and slenderer. Colony growth and expansion on the Pseudomonas agar medium showed three stages beginning with an initial slow stage (24 h), followed by a fast stage (48 h) and lastly a slow stage (72 h) (Figure 2). The colony spread observed on this medium was swimming dependent with colony growth and expansion occurring as distinct, expanding rings. Colony growth and expansion on the Kings B agar plates showed an initial slow stage up until ~ 24 h of incubation after which rapid growth and expansion occurred to nearly cover the plate (Figure 3). Colony growth and expansion on the LB agar increased gradually and steadily over the 72-h incubation period (Figure 4). However, compared to that observed on the nutrient agar the growth and expansion rate was faster. Several irregular swarming extensions appeared at different sites on the swimming colony boarders at 48 h resulting in further expansion from the primary streak culture across the plate. Colony growth and expansion on the nutrient agar medium increased gradually and steadily over the 72-h incubation period as shown in Figure 5. The growth observed on the nutrient agar medium appears to be non-motile which also explains the lack of colony expansion across the agar surface.

The results indicate that all the media used were suitable for the growth of *P. fluorescens* however the type and composition of the solid media used noticeably influence the colony morphogenesis of *P. fluorescens* as exhibited by distinct colony morphologies and growth patterns according to the culture media used (Table 3).

The motility, auto-aggregation, colour and pigment production were features that where visually verified to be the most strikingly affected (Table 4).

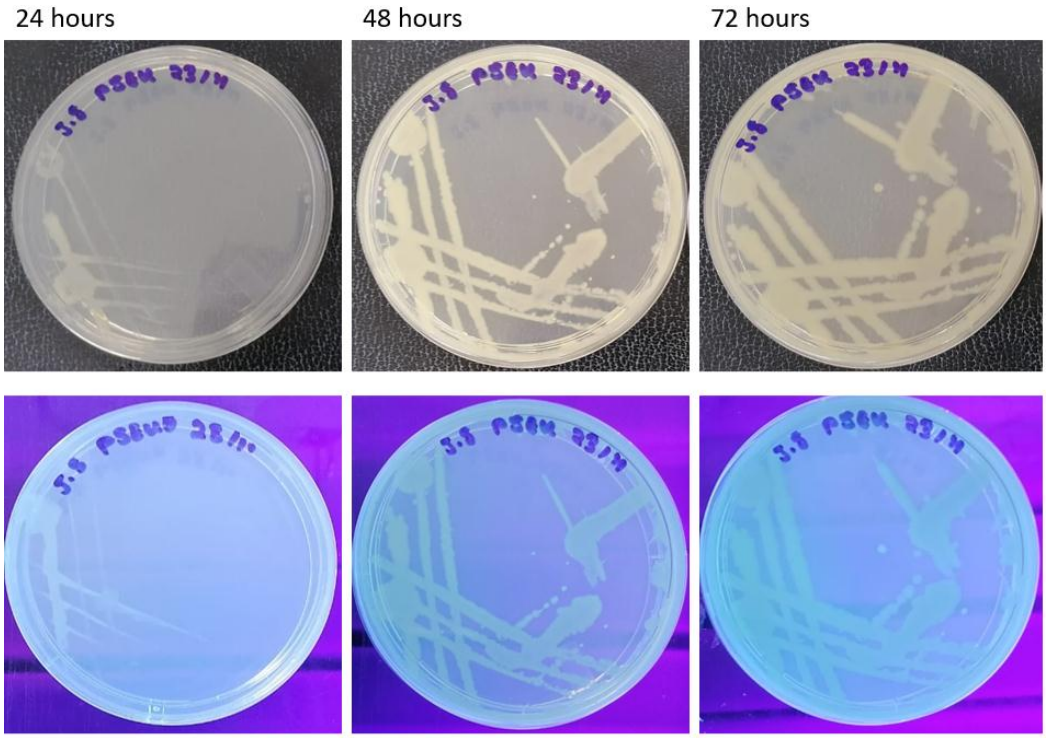

Figure 2. The (a) morphology and (b) fluorescens of PGPR *Pseudomonas fluorescens* on Pseudomonas agar over 3 days of incubation.
Source: Author

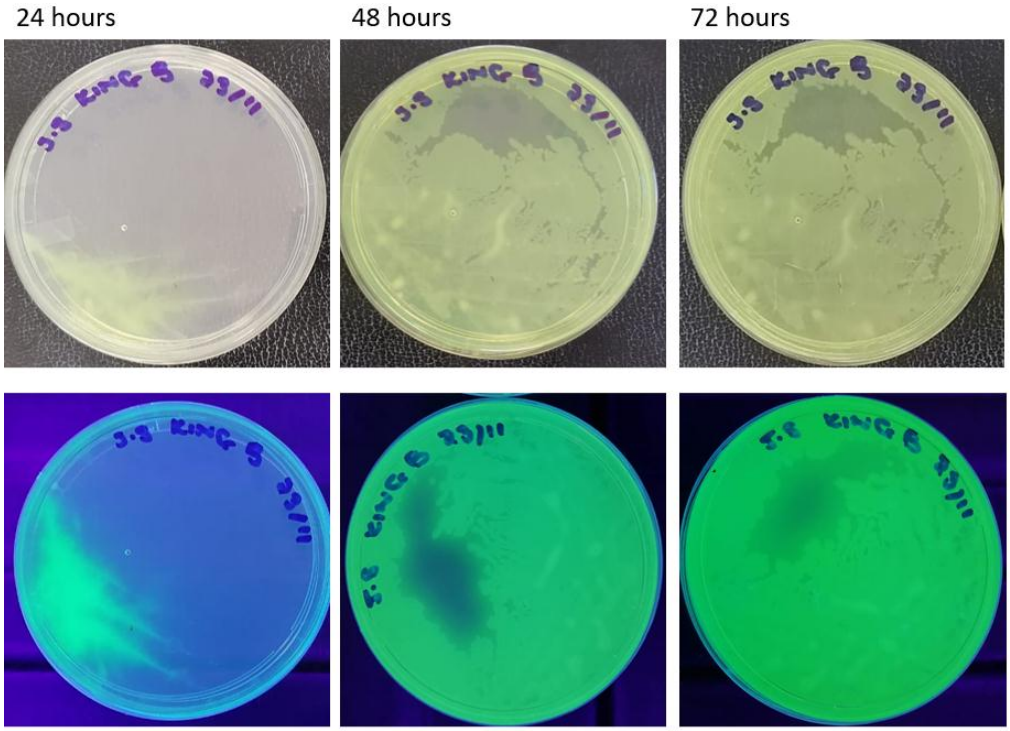

Figure 3. The (a) morphology and (b) fluorescens of PGPR *Pseudomonas fluorescens* on Kings B agar over 3 days of incubation.
Source: Author

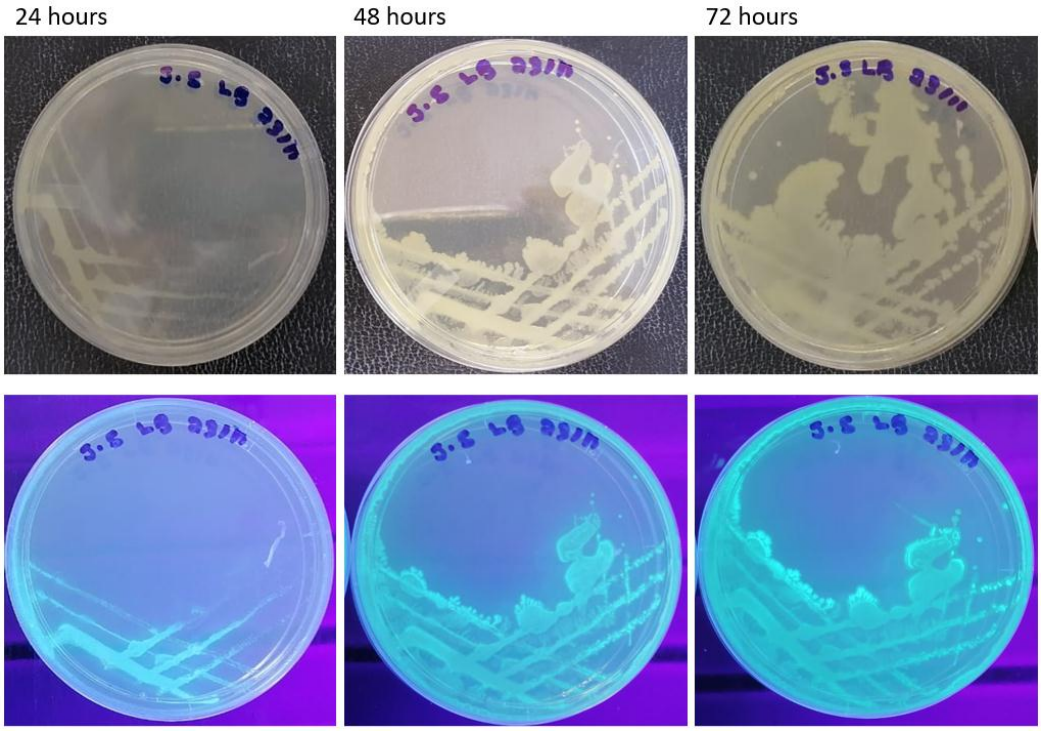

Figure 4. The (a) morphology and (b) fluorescens of PGPR *Pseudomonas fluorescens* on LB agar over 3 days of incubation.
Source: Author

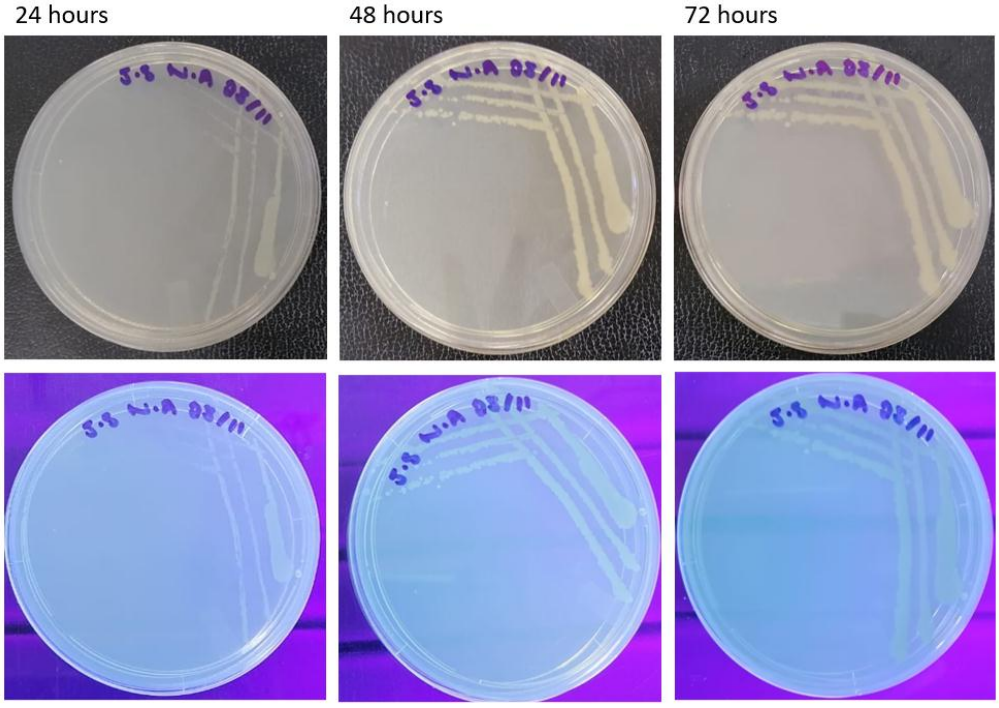

Figure 5. The (a) morphology and (b) fluorescens of PGPR Pseudomonas fluorescens on Nutrient agar over 3 days of incubation. Colony growth and expansion increased gradually and steadily over the 72-h incubation period.
Source: Author

Table 3. Effect of media composition on colony morphology and growth.

Colony morphology	Culture medium			
	Pseudomonas agar	**Kings B agar**	**LB agar**	**Nutrient agar**
Growth	Medium to fast growth	Fast growth	Fast growth	Medium growth
Form	Small, irregular colonies	Irregular, rhizoid, spreading, swarming colonies	Medium to large irregular, rhizoid colony shape, spreading or swarming colonies	Small, irregular and round colonies
Margin	Entire margin	Entire margin	Entire margin	Entire margin
Surface	Dull, smooth surface	Mucoid to glistening surface	Dull, smooth surface	Sull, smooth surface
Elevation	Slightly raised, convex	Slightly raised, convex	Slightly raised, convex	Slightly raised, convex
Opacity	Translucent	Translucent	Translucent	Translucent
Colour	Cream to dull yellow	Bright yellow to green	Cream to yellow	Cream to dull yellow
Mobility	Positive	Positive	Positive	Negative

Source: Author

Table 4. Effect of media composition on pigmentation and fluorescens.

Media	Pigmentation	Diffusible pigment	Fluorescens
Pseudomonas agar	+	-	++
Kings B agar	+++	+++	+++
LB agar	++	++	+++
Nutrient agar	+	-	+

+++ high/bright pigmentation/diffusible pigment/fluorescens; ++ medium pigmentation/diffusible pigment/fluorescens; + low/dull pigmentation/diffusible pigment/fluorescens; - no pigmentation/diffusible pigment/fluorescens
Source: Author

The results indicate that the PGPR *P. fluorescens* is a fast growing, nutritionally versatile, pigment producing, fluorescing bioinoculant that exhibits phase variation all of which strengthen its ability to survive, colonise and proliferate in the soil environment following introduction.

Abiotic stress tolerance and growth

The pH, temperature and salinity tolerance and growth limitations of the PGPR *P. fluorescens* are given in Table 5.

Growth was observed for a pH ranging between 5 (acidic) and 8 (alkaline), which indicates that it is not bound to a specific pH region and thus should not be sensitive to pH fluctuations above or below of neutral (pH 7) which are common in soil remediation methods. Extreme pH values of acidity and alkalinity would however impede the survival of this bioinoculant, regardless of liming and/or addition of alkaline amendments typically used during the rehabilitation of pH extreme sites. This biofertilizer exhibits temperature tolerance between 5 and 35 ± 2°C, which is sufficient for soil environments. Salinity tolerance was restricted to 4% NaCl, which allows the use of this biofertilizer to all but sodic soils. As sodic soils are not typically associated with metal sites or metalliferous mining areas this results only provides information for the few cases where the PGPR *P. fluorescens* should not be considered as a biological inoculant.

Heavy metal (HM) tolerance

The HM tolerance and growth limitations of the PGPR *P. fluorescens* are given in Table 6. The microbial inoculant

Table 5. Abiotic stress tolerance and growth results of PGPR P. fluorescens.

pH tolerance							
4	5	6	7	8	9	10	
-	+	+ (OG)	+	+	-	-	
I	T	T	T	T	I	I	
Temperature tolerance (°C)							
5	10	15	20	25	30	35	40
+	+	+	+	+ (OG)	+	+	-
T	T	T	T	T	T	T	I
Salinity tolerance (%)							
0.1	1	2	3	4	5	6	7
+ (OG)	+	+	+	+	-	-	-
T	T	T	T	T	I	I	I

+ Growth, - no growth; T tolerant, I intolerant; OG optimal growth.
Source: Author

Table 6. Heavy metal (HM) tolerance results of PGPR *P. fluorescens.*

Heavy metals	**Concentrations (ppm or µg/ml)**					**R (%)**
	10	**50**	**100**	**200**	**300**	
Iron	T	T	T	T	T	100
Manganese	T	T	T	T	T	100
Cobalt	T	T	T	T	T	100
Zinc	T	T	T	T	T	100
Copper	T	T	T	T	T	100
Lead	T	T	T	T	T	100
Chromium	T	T	T	I	I	60

T - tolerable; I - intolerable; R - resistance.
Source: Author

demonstrated 100% tolerance to all concentrations for all the HM tested except chromium, with concentrations > 200 ppm inhibiting growth. This finding once again allows us to identify contaminated soils and/or mine sites where the use of PGPR *P. fluorescens* would be suitable. As different soils and mine lands possess unique geochemical properties and heavy metal associations understanding of HM and the degree of their concentration effects will allow determining the usefulness of a particular microbial agent as the appropriate inoculants.

DISCUSSION

In context of developing countries such as South Africa environment-based biotechnologies being economically and technically feasible for economic growth and environmental preservation are urgently demanded. Despite this necessity, the environmental biotechnology market for industrial sector application in South Africa is absent. Developing-country limitations to national environmental biotechnological capabilities must be acknowledged and the development of novel and leading environmentally sound technology undertaken in accordance.

For decades the introduction of desired bacteria and fungi into the soil has occurred for agricultural purposes (Chaudhary et al., 2020). These inoculations have been done to stimulate plant growth, supply nutrients, improve the structure of soil and control plant pathogen activity (Chaudhary et al., 2020). In more recent years, there have been other objectives for the introduction of microbial organisms into the soil (Rizvi et al., 2022). These include the bioaccumulation of inorganic compounds, microbial leaching and the bioremediation of pollutants in the soil (Nadeem et al., 2016).

Successful soil augmentation requires the selection of

a microbial inoculant that is easily cultured; fast growing; occur and survive in a wide range of environmental conditions and can withstand high concentrations of contaminants (Mrozik and Piotrowska-Seget, 2010). The survival of strains introduced into the soil is one of the major difficulties associated with bioaugmentation procedures (Thompson et al., 2005). The effectiveness of bioaugmentation is influenced by both biotic and abiotic factors and the effect the have on the survival of the introduced microorganisms (Bento et al., 2005). Moreover, the efficiency of bioaugmentation is also determined by the soil type; nutrient content and aeration (Bento et al., 2005). Being an *in-situ* treatment, bioaugmentation, provides a safe and economic alternative to commonly used physicochemical strategies (Adams et al., 2015).

Insight into microorganisms displaying multifaceted beneficial traits such as plant growth promotion, rhizosphere competence, abiotic stress tolerance and/or heavy metal stress tolerance is of great importance in the realm of environmental biotechnology. Such microorganisms present novel agents in regulated and legally obligated ecological restoration efforts. The prospect of identifying and make use of already available and commercially sold bioproducts for environmental applications other than in agriculture is significant, especially in developing countries where research and funds restrict remediation practices. To the best of our knowledge this study is the first of its kind with regards to exploring the bioremediation potential of agronomic products sold in South Africa.

Phenotypic or phase variation is an important characteristic demonstrated in rhizosphere competent bacteria as it allows the achievement of population diversification within a species crucial in niche adaptation and enhance bacterial fitness when subjected to certain unfavourable conditions (Van den Broek et al., 2005). Colony morphotyping has been shown to be a definitive technique for assessing phenotypic variation which is an important measure colonization, survival and adaptive competence in the soil system (Sousa et al., 2013). The colony morphology method was applied in this study as means of rapidly predicting the competence of a selected strain and therefore its enduring performance following introduction into the field.

During colonisation of the different media, *P. fluorescens* underwent phenotypic variation which resulted in the emergence of colonies having different morphologies. The appearance of colony morphology variation of the *P. fluorescens* on and between each of the different media, representing structured environments, demonstrates the capability for both stress tolerance and niche specialisation. Additionally, the diffuse morphology observed for some colonies indicates that motility on the agar substrates may have occurred. This is not an unusual observation as *P. fluorescens* possess flagella required for swarming and swimming (Santoyo et al., 2021). Mobility has several advantages related to rhizosphere competence as it allows the microbe to colonise a greater area of the rhizosphere and populate potentially less microbially dense parts under competitive stress (Sánchez-Contreras et al., 2002). Colony colour and pigmentation variation was also observed for *P. fluorescens* on the different agar substrates. Under UV light the *P. fluorescens* demonstrated a blue pigmentation and fluorescence on the *Pseudomonas* agar, LB and nutrient agar and a vibrant green on Kings B. Pigmentation and fluorescence appears to be linked to the nutritional composition of the growth media. Pigment production is an important parameter as it is correlated to stress tolerance and survival under different environmental conditions (Ahmad et al., 2016).

Environmental stress is a major challenge for the sustainability of rehabilitation and therefore ecosystem recovery of mine sites, soils and rock wastes (Goswami and Deka, 2020; Khan et al., 2020). For a microbial inoculant to be considered effective in stress environments it is necessary that they display the ability to survive in adverse conditions. The results of this study suggest that the commercially available PGPR *P. fluorescens* demonstrates such a capacity for survival and persistence in unfavourable abiotic environments further supporting it suitability in applications of bioremediation.

The global repositioning to a greener economy and the subsequent biotechnological potentials of heavy metal tolerant microbes and their cellular products provided major momentum into their research. In general, microorganisms have many beneficial applications in the environment and are in large used for agricultural purposes such as promoting crop yields and/or increasing soil fertility (Nazli et al., 2020). However, biotechnological processes in large require that for microbial species to play a significant role, especially in matters regarding environmental and ecosystem recovery and/or heath, they need to demonstrate heavy metal tolerance (Nazli et al., 2020). Our study shows that PGPR *P. fluorescens* is of 100% tolerance to all heavy metals tested except for chromium. However, the extent of growth was affected by increasing heavy metal concentrations for all metals tested implying increased sensitivity to higher metal concentrations. Apart from chromium the heavy metal tolerance of the PGPR *P. fluorescens* strain indicates that they could survive and have bioremediation potential for HM-polluted environments. Tolerance to heavy metals by certain PGPR microorganisms indicates their suitability as bioinoculants to be applied to contaminated environments in terms of their probable survival. Based on the demonstrated heavy metal tolerance PGPR *P. fluorescens* strain should be further investigated for the possibility of being used for soil bioremediation purposes in heavy metal contaminated soils.

Conclusion

The success of using microbial agents as bioinoculants for soil bioremediation technologies will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms. The use of commercially available PGPR *P. fluorescens* as a bioinoculant for the bioaugmentation and bioremediation of contaminated soils relies on its ability to survive, replicate and perform the desired biotechnological appointment. Rhizosphere competence, abiotic stress and heavy metal tolerance to certain common environmental parameters associated with mine lands and/or contaminated soils enables the survivability of the microbial inoculant to be gauged. This study showed that common, widely applied and available agronomic biofertilizers, *P. fluorescens*, exhibits an exceptional capacity to withstand hostile environmental conditions which in addition to its numerous plant growth promoting traits, make it a desirable microorganism using in environmental biotechnologies applied to ecosystem restoration.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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