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Comparison of Meristem Culture and Heat Therapy to Clean Garlic (*Allium sativum* L.) Infecting Virus in Ethiopia

Abel Debebe
Ethiopian Institute of Agricultural Research, Melkassa Agriculture Research Center
P.O. Box 436, Adama, Ethiopia, E-mail aabbeelldebebe@gmail.com

አህፅርኦት

የዚህ ጥናት ዓላማ ሶስት የተሻሻሉ የነጭ ሽንኩርት ዝርያዎችን የሚያጠቃ የቫይረስ ዓይነትን መለየትና በሽታዎችን ማፅዳት ነው። የዝርያዎቹ ኮረቶች በናይሮቢ ኬንያ በሚገኘው ሥነ-ሳይንስ እና ዓለም ዓቀፍ የቀንድ ከብቶች ምርምር ተቋም ውስጥ በሚገኘው የጥላ ዳስ በ2006 ዓ.ም. ከተተከሉ በኋላ ለጋ ቅጠል እስኪያወጡ ድረስ በእንክብካቤ ተይዘዋል። ጥናቱ እንዳሳየው ፖቲ ቫይረስ ዋናው ቫይረስ ሆኖ ተገኝቷል። ፖቲ ቫይረስን ለመለየት የሚያስችሉ ሁለት ፕራይመሮች በስም ለመጥቀስ CP እና Nib በመጠቀም ሪቨርስ ትራንስክሪፕቴስ ፖሊመሬስ ቼን ሪያክሽን (RT-PCR) ዘዴን በመከተል ቫይረሶቹን የመለየት ስራ ተከናውኗል። ለዚህ ጥናት የዋሉት ፕራይመሮች የተዋቀሩት ከኢትዮጵያ በተገኙ በቫይረስ የተበከሉ የነጭ ሽንኩርት ናሙናዎችን ጥልቅ ሲኮንስ(deep sequence) በማድረግ በተገኘ መረጃ ነው። በሪቨርስ ትራንስክሪፕቴስ ፖሊመሬስ ቼን ሪያክሽን (RT-PCR) ውጤት መሰረት ሶስቱም የተሻሻሉ ዝርያዎች መዶ በመዶ በፖቲ ቫይረስ መጠቃታቸው ተረጋግጧል። ዝርያዎቹን ለማፅዳት ይቻል ዘንድ ሁለት መንገዶች ማለትም መርስተም ካልቸር (meristem culture) እንዲሁም ከሶስቱም ዝርያዎች በቫይረስ የተበከሉ ኮረቶችን በሙቀት ቻምበርስ/Growth Chambers/ ውስጥ በ38^{0}c ለ60 ቀናቶች ከተቀመጡ በኋላ መርስተሙን በመለየት ሙራሺግ እና ስኩግ (Murrashiage and Skoog) ውህድ ላይ ካልቸር አድረጎ ለማወዳደር ተሞክሯል። የጥናቱ ውጤቱ እንደሚያስገነዘበው 77 `በመዶ የሚሆኑት ከመርስተም ካልቸር ያደጉ የላቦራቶሪ አፅዋቶች ከፖቲ ቫይረሱ መንፃት ሲችሉ፤82 በመዶ የሚሆኑ ከቫይረስ የፀዱ የላቦራቶሪ አፅዋቶች መርስተም ካልቸርን ከሙቀት ጋር በመቀላቀል ለማግኘት ተችሏል። በመጨረሻም የዚህ ሙከራ መረጃ ከቫይረስ የፀዳ የነጭ ሽንኩርት ለማምረት የሚያስችል አንዱ ዝብአት በመሆን ጥራቱን የጠበቀና ከፍተኛ የነጭ ሽንኩርት ምርት እንዲኖር ይረዳል።

Abstract

*Experiment to detect garlic-infecting virus for three improved garlic varieties and to clean the virus were conducted in Ethiopia. Garlic cloves were planted in screen house at Biosciences Eastern and Central Africa-International Livestock Research Institute Hub Nairobi, Kenya in 2014. Reverse Transcription polymerase chain reaction (*RT-PCR) technique was applied using two general Potyvirus detecting primer sets designated as CP and Nib. The primers were designed based on deep sequencing information previously generated from infected Ethiopian garlic samples. The RT-PCR diagnosis showed that the three improved garlic varieties were completely infected by potyvirus across all tested sites. Thus, to recover the cultivars two techniques viz. meristem culture alone and meristem culture associated with thermotherapy (cloves treated with 38^{0}c for 60 days) were compared. Then, indexing by RT-PCR indicated that 77% and 82% in vitro plantlets were found to be virus free from meristem culture alone and thermotherapy associated with meristem culture, respectively.*

Introduction

Garlic (*Allium sativum L.*) is the most important and widely cultivated *Allium* used for flavoring and medicine throughout ancient and modern history of human (Abdullah *et al*., 1988). . It is grown all over the world from temperate to subtropical climate but China is the largest producer (Mishra *et al*., 2014). According to Martin *et al*. (1995) and Conci *et al.* (2003) bulbs of garlic harbor complex of virus due to its exclusive vegetative propagation which in turn results in yield and quality reduction of about 50-78%. Twelve major viruses from three genera viz. *Potyviru*s (family, *Potyviridae*), *Allexiviru*s (family, *Alexiviridae*) and *Carlavirus* (family, *Betaflexiviridae*) had been identified as the main infection agents (Parrano *et al*., 2012).

In Ethiopia, garlic is the second most widely cultivated *Allium* crop produced in Adet, Ambo, Debere-work, Sinana, Jimma, and other Ethiopian highlands (Worku & Dejene, 2012). . Despite its importance, garlic production has been seriously challenged by a biotic and biotic stress Kero (2010). Virus is one of the most important constraints in garlic production in the world. However, very little information is available on garlic infecting viruses and cleaning attempts

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Several research reports indicated that meristem shoot tip culture either alone or with the combination of thermotherapy had been used as the most effective virus eradication method to get high yield and quality plant propagules (Conci *et al*., 1992; Martin *et al*., 1995; Fajardo *et al*., 2001; Conci *et al*., 2003). Thus, generation of comprehensive information to evaluate the occurrence of viruses in released garlic cultivars and eliminating the viruses using meristem culture and thermotherapy could be considered as a base line to establish virus free garlic dissemination scheme in Ethiopia.

Therefore, this experiment was initiated with the objectives of assessing the prevalence and incidences of garlic viruses in released garlic cultivars using RT-PCR and cleaning the viruses from the improved cultivars through heat therapy and meristem culture

Material and Methods

Three improved varieties; **Bishoftu nech, Tseday** and **Kuriftu** released by the Deber Zeit Agricultural Research Centre, Ethiopia were used for this study. Ten cloves per cultivars were planted in pot using soil mix prepared in a ratio of 2:1 forest soil and sand at Biosciences Eastern and Central Africa International Livestock Research Institute (BecA-ILRI) Hub, Nairobi Screen house in 2014.

After thirteen days of planting the cloves, young leaves which showed yellowing, mosaic and stunting symptoms were collected for RNA extraction. Total RNA was extracted from each sample using ZR plant RNA Mini Prep kits following the manufacturer procedures (www.zymoreserch.com). The RNA quantity and purity was evaluated using NanoDrop Spectrophotometer. First strand cDNA synthesis was performed following the procedure of Maxima H minus first strand cDNA synthesis kit. Two sets of general primer pairs designated as (CP and Nib) were used for the detection of *Potyvirus.*

The primers which were used for this experiment were designed based on coat protein and nuclear inclusion protein gene sequence information from deep sequencing of Ethiopian garlic infected samples. Amplification of cDNA was performed with BIONEER PCR reaction mix with template DNA, forward and reverse primer pairs for targeting general *Potyvirus.* The temperature profile for the PCR using CP primer pairs was 94^0C for 3 min, 30 cycles (94^0C 30 sec, 61^0C for 1 min and 72^0C for 1 min) and final extension of 72^0C for 7 min. The same profiles were applied for the other primers by only changing the appropriate annealing temperatures for the primers (Table 1). The PCR products were analyzed by Gel Electrophoresis with 1.5% Agarose gel run for 40 min at 100 Volts. The presence or absence of approximate amplicon size bands of 350bp for Cp and 520 bp for Nib was recorded (Fig.1).

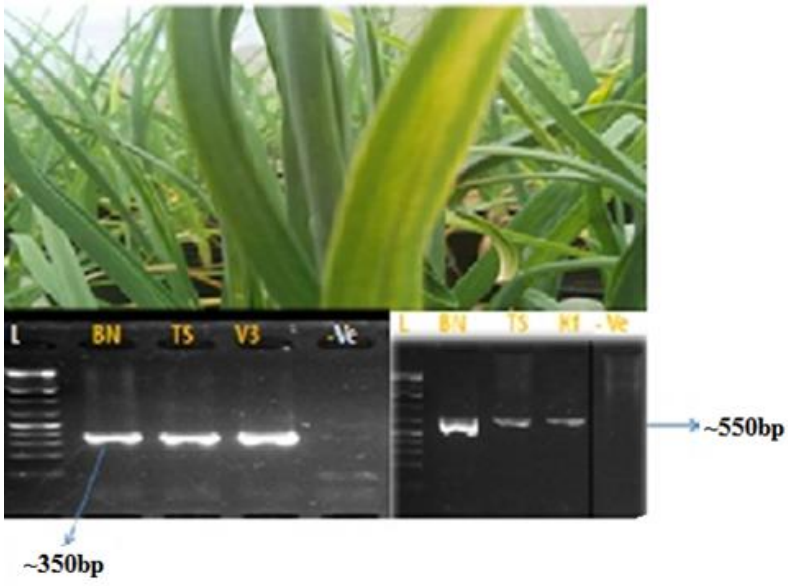

Figure 1. Indexing of garlic viruses from screen house grown young seedling of improved varieties of garlic (*L-100bp ladder, BN-Bishoftu nech, TS-Tseday and kf-Kuriftu, infected improved varieties)

Table 1. Primers pairs used for the amplification of coat protein (CP) and nuclear inclusion protein (Nib) genes of potyvirus

Primer	Sequence (5'-3')	TA (^{0}C)	TM (^{0}C)	Exp. Amp (bp.)
Potyvirus-General				
CP_{For}	TGG ACT ATG ATG GAT GGC GTG GA	61	55.8	350
CP_{Rev}	TGT GTG CCT YTC CGT GTC CT		57.9	
Nib_{For}	CCA AAA CTA GAT CAA GAG CG	56	49.1	550
Nib_{Rev}	TCG CCA TCC ATC ATA GTC C		52.7	

* Cp-designated coat protein, Nib- nuclear inclusion protein,GarVb-Garlic virus b,GarVc- Garlic virus c and GarVd- Garlic virus d, TA- annealing temperature, TM-melting temperature, Exp.amp-expected amplification

After identifying the *potyvirus* virus from the first experiment, two consecutive trials were established to clean the three improved cultivars in which half of the cloves were exposed to heat at 38^{0}C for 60 days and the remaining half were kept at room temperature as control. Forty heat-treated and forty untreated bulblets per variety were used for the procedure. Two hundred forty experimental units for the three varieties were established. The bulblets were disinfected with 2% sodium hypochlorite for 20 minutes and rinsed 3 times with sterilized distilled water. after finalizing the disinfection in the hood, microscopic meristem dome with two leaves primordial were excised and cultured in test tubes containing 15 ml of mineral salt media in addition to 3% sucrose, 0.2% gelrite, and 100 mg.L^{-1} of : myo-inositol,; 1 mg.L^{-1} of Thiamin HCl, and without addition of growth hormone (Murashige and Skoog 1962). The test tubes were placed in growth room at 25^{0}C with a photoperiod of 16 hour, after six weeks of culture initiation, rate of shoot regeneration from the explants and efficiency of the techniques for the elimination of the viruses were compared. In vitro leaves from 30 test tubes per variety and 15 from each treatment were taken. Totally, 90 samples were taken for RNA extraction. The RT-PCR diagnoses for the in vitro plantlets were carried out by using *Potyvirus* targeting primers.

Result and Discussion

The improved cultivars were 100% infected by *Potyvirus*. Therefore, validation of the elimination techniques evaluated for cleaning the *Potyvirus.*

The establishment percent of meristems extracted from heat treated and untreated cloves showed different response. For the three cultivars, 60% of the meristem explants were regenerated from cloves, which had not been exposed to thermotherapy. Whereas, the survival rate of meristem explants from heat-treated cloves was 90% (Table 2). In general, the number of established in vitro plantlets was higher in heat-treated bulbs than untreated ones. Similar result has

been reported by Szyndel *et al. (*1994) who indicated that heat-treated bulbs gave better establishment.

Table 2. Results of heat treated and untreated cloves of shoot tip culture on survival rate of meristem

Cultivar	Explant source	No. of explant initiated	No. of plantlet regenerated	% of plantlet established
Bishoftu nech	Untreated Cloves	40	23	58
Tseday	Untreated Cloves	40	22	55
Kuriftu	Untreated Cloves	40	28	70
Total		120	73	60
Bishoftu nech	Treated Cloves	40	35	88
Tseday	Treated Cloves	40	37	93
Kuriftu	Treated Cloves	40	36	90
Total		120	108	90

**Bn,Ts &ku are improved cultivars*

Tabel 3. Efficiency of combination of thermotherapy with meristem culture as compared to meristem culture alone for virus cleaning

Cultivar	Explant source	No. of In vitro plant tested	No. of virus free plantlet	% of virus plantlet
Bishoftu nech	HT Cloves	15	14	93
Tseday	HT Cloves	15	12	80
Kuriftu	HT Cloves	15	12	80
Total		45	38	84
Bishoftu nech	Untreated Cloves	15	11	73
Tseday	Untreated Cloves	15	13	87
Kuriftu	Untreated Cloves	15	10	67
Total		45	34	75

HT-Heat treated cloves

Result of indexing the invitro plantlets for *Potyvirus* showed that the two cleaning techniques gave different response in which 84% of the in vitro plantlets initiated from heat-treated cloves were found to be virus free. Whereas, 75% of in vitro plantlets for the three cultivars which were extracted from untreated cloves were found to be virus-free (Table 3 and Fig. 2). In general, thermotherapy and meristem culture gave better efficiency for virus cleaning. Similar results have been reported by (Robert *et al*., 1998; Torres *et al*., 2000; Panattoni *et al*., 2013). According to Murashige (1974) the mechanism of virus elimination had been explained by in activating viruses and/or inhibited virus multiplication. Furthermore, shoot tip culture is the most commonly used method to produce virus-free plants. Moreover, several research reports indicated that virus cleaning using meristem culture either alone or with the combination of thermotherapy had been applied and found to be the most effective methods to get high yield and quality plant propagules (Conci *et al*., 1992; Fajardo *et al*., 2001and Conci *et al*., 2003). Ultimately, in this study the RT-PCR assay and the technique to recover infected improved garlic cultivars were found to be effective, and thus, this protocol and research finding could be transferred and applied to establish virus-free garlic dissemination scheme in Ethiopia.

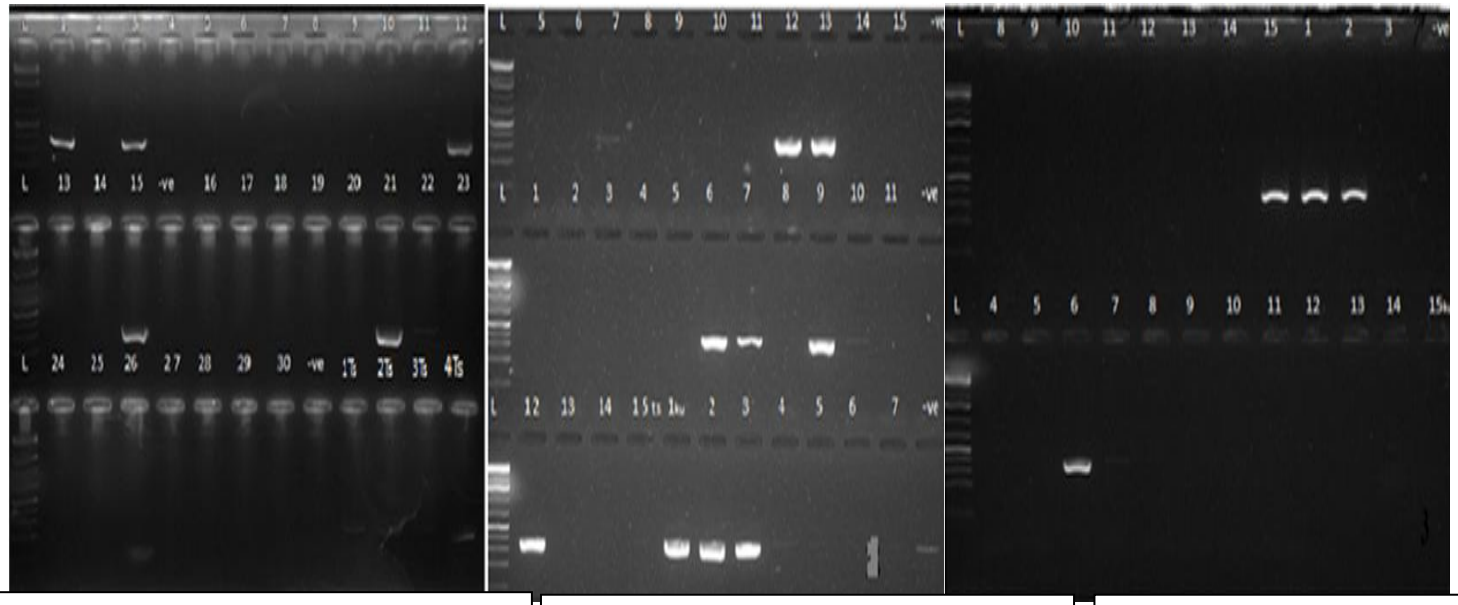

Fig 2a. RT-PCR for variety BN (lane L is the size marker, lane 1to 15 meristem cultured invitro plantlets, while lane 16-30 shows invitro plantlets from heat treatment plus meristem culture

Fig 2b. RT-PCR for variety TS (the last four lanes from fig 2a up to lane 15 in this figure shows meristem cultured in vitro plantlets, while lane 1-15 in the second row shows invitro plantlets from heat treatment plus meristem culture

Fig 2c. RT-PCR for variety Ku (the last seven lanes from fig 2b and lane 8- 15 in this figure in first row shows meristem cultured in vitro plantlets, while lane 1-15 in this fig shows invitro plantlets from heat treatment plus meristem culture

Figure 2. Indexing the garlic cultivars after meristem culture alone and meristem culture combine with thermotherapy techniques using RT-PCR

Conclusion

The result of this study showed that the three improved garlic cultivars were totally infected by the virus. The protocol for recovering the improved cultivars provides successful results.Ultimately, the knowledge and protocol tested and verified in this study could be transferred and applied to garlic production system in Ethiopia to establish virus free garlic dissemination scheme.

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