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# *In vitro* regeneration and molecular characterization of some varieties of *Lycopersicon esculentum* Mill. in Bangladesh

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**Abstract**

An efficient regeneration protocol was established for two varieties (BARI tomato-9 and BARI tomato-15) of tomato (*Lycopersicon esculentum* Mill.) using three explants namely cotyledonary node, cotyledonary leaf and hypocotyls. Among the three explants, maximum number of shoots was produced from cotyledonary leaf explants of BARI tomato-15 on MS with 2.0 mg/l BAP and 0.5 mg/l IAA. In this combination of BAP and IAA 86%, on an average, cotyledonary leaf explants showed regeneration response 14.12 shoots/explants. Explants from hypocotyl showed best results in MS medium with 2.0 mg/l BAP and 0.2 mg/l IAA in both the varieties. In case of cotyledonary node, BARI tomato-15 showed 6.0 shoot/explant on MS with 2.0 mg/l BAP and 1.0 mg/l IAA. Molecular characterization of total ten varieties of tomato in Bangladesh was done by using six arbitrary oligonucleotide RAPD primers. A total of 140 bands were produced where the highest genetic distance (0.6769) was found between BARI tomato-3 and Mintoo tomato and lowest distance (0.1035) was observed between BARI tomato-7 and BARI tomato-8. This result will be useful for designing future breeding programs.

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**Keywords:** *Lycopersicon esculentum;* Leaf explants; Multiple shoot; RAPD analysis

## Introduction

Tomato (*Lycopersicon esculentum* Mill., Fam.: Solanaceae) is one of the most important edible and nutritious vegetable, native to Central and South America. It contains protein, lipid, carbohydrate, mineral and fiber. Tomato is a rich source of minerals, vitamins and organic acid, essential amino acids and dietary fibers. It is extensively cultivated throughout the world. In Bangladesh, it is cultivated in almost all kitchen gardens and also in the fields for its adaptability to wide range of soil and climate (Hossain *et al*., 2010). Due to its popularity, Bangladesh Agricultural Research Institute (BARI) has made several breeding programs to improve desirable characters such as fruit size, color, disease resistance, etc. in new tomato varieties. As a result, 13 varieties and 8 hybrid varieties of tomato were released by BARI (www.bari.gov.bd). In Bangladesh tomato cultivation and production is hampered due to various biotic and abiotic stresses. In overcoming such constraints of tomato production, breeding and biotechnological techniques have been applied elsewhere (Oktem *et al*., 1999). Breeding program associated with biotechnological tools depends upon the development of an efficient *in vitro* plant regeneration system (Abu-El-Heba 2008). Molecular markers have become important tools in studying genetic diversity (Bered *et al.,* 2005). Random amplified polymorphic DNA (RAPD) analysis through the polymerase chain reaction (PCR) has been widely used in molecular characterization and traces the phylogeny of diverse plant. Genetic information has been considered as an important factor and pre-requisite for plant improvement program (Chaudhuri *et al*., 1976). Since the morphological characterization does not provide accurate information necessary to distinguish different genotypes, further assessment of collected germplasms at the molecular level is required (Carmen de Vicente *et al*., 2006). The present study was conducted to test an efficient regeneration protocol and the genetic diversity of the local tomato varieties of Bangladesh using RAPD markers.

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**Materials and methods**

In this study, two varieties of tomato namely, BARI tomato-9 and BARI tomato-15 were used for *in vitro* regeneration. While for molecular characterization, 10 other varieties namely, BARI tomato-2, BARI tomato-3, BARI tomato-7, BARI tomato -8, BARI tomato -9, BARI tomato-14, BARI tomato-15, Mintoo tomato, Delta tomato and Sawsan tomato were used. For the preparation of explants surface sterilized procedure were followed according to the protocol describe by Khan *et al*. (2017). The sterilized seeds were then transferred in autoclaved cotton soaked bottle for *in vitro* germination and growth of seeds. Different explants such as cotyledonary leaf, cotyledonary node and hypocotyls were excised from 8-10 days old seedling and inoculated on MS (Murashige and Skoog, 1962) media containing BAP, Kn and IAA, singly or in combinations for *in vitro* regeneration of shoots. Cultures were sub-cultured to fresh media regularly, at an interval of three to four weeks for maintenance. All cultures were maintained under fluorescent light of 20,000-25,000 lux intensity on a 16/8 (light/dark) hours at 25 ± 2°C. For induction of roots, regenerated shoots (2.5 – 4.0 cm long) were excised and transferred to MS and half strength MS medium with 3% sucrose without hormonal supplements. The plantlets with well-developed root system were transplanted in sterilized soil in small pots.

For RAPD analysis genomic DNA were isolated from fresh leaves of the 10 tomato varieties using CTAB method (Doyle and Doyle 1987). DNA is quantified by 0.8% (W/V) agarose gel electrophoresis and spectrophotometer (Analylikjena, Specord 50, Germany), respectively (Naz *et al.* 2013). Six RAPD primers such as OPA-1, OPA-4, OPA-5, OPA-10, Primer-6, and Primer-12 were used for RAPD analysis of 10 tomato varieties. Polymerase chain reactions (PCRs) were performed in 25 µl of reaction mixture containing Taq buffer A 10x (10 mM Tris-HCl with1.5 mM $MgCl_2$) 2.5 µl, primer (10 µM) 1.0 µl, dNTPs mix (10 mM) 0.5 µl, Taq DNA polymerase (5 U/µl) 0.2 µl, Template DNA(25 ng) 2 µl and sterile de-ionized distilled water 18.8 µl.

DNA amplification is carried out in Thermal cycler and amplification products are separated by horizontal electrophoresis in agarose with ethidium bromide (10 mg/ml). DNA bands were observed on UV-transilluminator and photographed by a gel documentation system (ms major science UVDA). The photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. The scores obtained using all primers in the RAPD markers analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the germplasm using computer program "POPGENE 32" (Version 1.32).

**Results and discussion**

The present investigation was firstly aimed to establish an efficient regeneration protocol of two BARI tomato varieties namely BARI tomato-9 and BARI tomato-15. MS supplemented with various hormones were applied for induction of shoots from various explants such as cotyledonary leaf and node and hypocotyl. Multiple shoot formation with difference in numbers was observed in all hormonal treatments (Table I). Cotyledonary node showed direct organogenesis, where hypocotyls and cotyledonary leaf explants showed indirect shoot regeneration response.

In the present study, out of the three explants of tomato cotyledonary leaf showed maximum multiple shoot regeneration (average 14.12 shoots/explants) on MS with 2.0 mg/l BAP and 0.5 mg/l IAA in BARI tomato-15 where 86% of explants responded ( Table I, Fig. 3 A and B). On the other hand, BARI tomato-9 showed best response on MS with 2.0 mg/l BAP and 1.0 mg/l IAA from same explants with lower mean number (5.0) of shoots (Table 1).There are several reports of using BAP and IAA for shoot regeneration from cotyledonary leaf explants of tomato (Sarker *et al*. 2009, El-siddig *et al*. 2009).

When MS with 2.0 mg/l BAP and 0.2 mg/l IAA were used 100% hypocotyl explants responded towards regeneration which was the best combination for hypocotyls in both the varieties of tomato (Fig 3C-D). In this combination of BAP and IAA average number of shoots was varied in two varieties of tomato where average 6.5 shoots/explants was obtained in BARI-9 and 9.2 shoots/explants in BARI-15 (Table 1). Kanakapura and Pradeep (2013) found best direct shoot regeneration on MS with 2.0 mg/l BAP and 0.1 mg/l IAA from hypocotyls explants of tomato. In case of cotyledonary node explants MS with 2.0 mg/l BAP and 0.2 mg/l IAA showed best response towards regeneration in BARI tomato-9 where 100% explants responded towards shoot initiation (Fig 3 E) and mean number of shoots/explants was 4.2 (Table 1). On the other hand, BARI tomato-15 showed 100% regeneration response from cotyledonary node explants on MS with 2.0 mg/l BAP and 1.0 mg/l IAA with 6.0 average number of shoots/explants (Fig. 3F, Table 1). Kanakapura and Pradeep (2013) reported that MS with BAP 4.0 mg/l and IAA 1.0 mg/l combination was the best for cotyledonary node and cotyledonary leaf explants of tomato.

**Table I. Effect of different hormonal supplements on MS medium for *in vitro* shoot regeneration from various explants of BARI tomato-9 and BARI tomato-15.**

| Hormonal supplement (mg/l) | | | | Explants | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| BAP | Kn | IAA | Variety | Cotyledonary node | | Cotyledonary leaf | | Hypocotyls | |
| | | | | % of responsive explants | Mean number of shoots per explants | % of responsive explants | Mean number of shoots per explants | % of responsive explants | Mean number of shoots per explants |
| 2.0 | 0.5 | - | BT-9 | 90 | 2.15 | 54 | 4.15 | 87 | 5.13 |
| 2.0 | 0.5 | - | BT-15 | 60 | 3.33 | 70 | 5 | 66.6 | 4.5 |
| 2.0 | 1.0 | - | BT-9 | 83 | 2 | 46 | 4 | 74 | 4.5 |
| 2.0 | 1.0 | - | BT-15 | 100 | 2.00 | 63 | 5 | 80 | 4 |
| 2.0 | 1.5 | - | BT-9 | 75 | 2 | 34 | 4 | 80 | 4 |
| 2.0 | 1.5 | - | BT-15 | 83 | 2.22 | 60 | 4.5 | 53.3 | 4 |
| 2.0 | 2.0 | - | BT-9 | 83 | 1.5 | 40 | 3 | 90 | 4.8 |
| 2.0 | 2.0 | - | BT-15 | 100 | 2.16 | 40 | 4.0 | 70 | 3.6 |
| 2.0 | 0.5 | 0.5 | BT-9 | 86 | 3 | 80 | 4.8 | 100 | 4.8 |
| 2.0 | 0.5 | 0.5 | BT-15 | 100 | 3.20 | 86 | 3.8 | 73 | 4 |
| 2.0 | 0.5 | 1.0 | BT-9 | 100 | 3.5 | 100 | 5.2 | 86 | 4.5 |
| 2.0 | 0.5 | 1.0 | BT-15 | 100 | 4.00 | 86 | 4.0 | 100 | 5.2 |
| 2.0 | - | 0.2 | BT-9 | 100 | 4.2 | 40 | 3.2 | 100 | 6.5 |
| 2.0 | - | 0.2 | BT-15 | 100 | 4.00 | 80 | 7.4 | 100 | 9.2 |
| 2.0 | - | 0.5 | BT-9 | 100 | 3.3 | 50 | 4.5 | 86 | 4.3 |
| 2.0 | - | 0.5 | BT-15 | 100 | 5.50 | 86 | 14.12 | 100 | 6.42 |
| 2.0 | - | 1.0 | BT-9 | 100 | 3 | 60 | 5 | 86 | 5 |
| 2.0 | - | 1.0 | BT-15 | 100 | 6.00 | 80 | 12.25 | 100 | 7.2 |

BT-9 = BARI tomato-9; BT-15 = BARI tomato-15

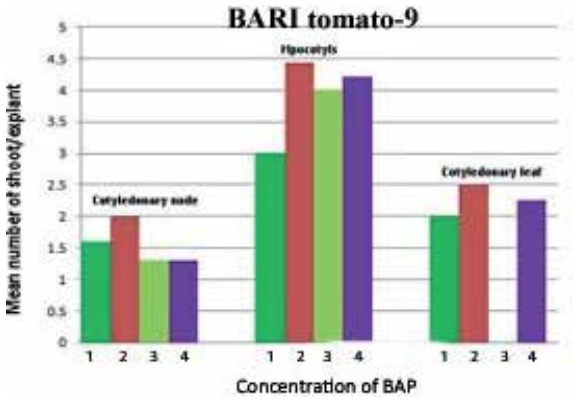

Fig. 1

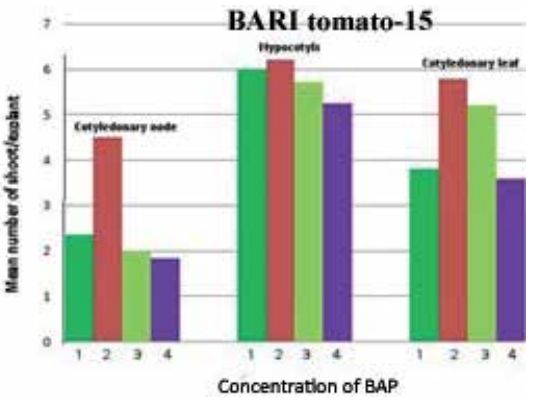

Fig. 2

**Figs. (1-2). Effect of different concentration of BAP (1-4 mg/l) with MS medium for the regeneration of different explants of two BARI tomato varieties**

In the present study, different concentrations of BAP (1-4 mg/l) were used for regeneration of two BARI tomato varieties. MS medium with 2.0 mg/l BAP was the most effective combination for adventitious shoots formation in all the explants and the varieties (Fig. 1-2).

Among the three explants hypocotyl showed best result in this combination of BAP (Fig. 3G and H) where mean number of shoots per explants was 6.2 and 4.45 in BARI tomato-15 and BARI tomato 9 respectively which support the results of Mohamed *et al*. (2010).

Effects of BAP, Kn and IAA were examined on multiple shoot regeneration from three explants of tomato (Table-I). Among the three explants cotyledonary leaf of both the varieties showed best response towards shoot regeneration (100%) on MS with 2.0 mg/l BAP + 0.5 mg/l Kn + 1.0 mg/l IAA with 5.2 average shoots/explant ( Fig 3I). Use of low dose of IAA (0.5 mg/l) with moderate dose of Kn (2.0 mg/l) was found to be optimum for enhanced plantlet regeneration (Locky 1983). However Kartha *et al*. (1977) suggested that high levels of IAA were found to be promising probably due to genotypic and explants specificity. In the present study it


Fig. 3. (A-L). Regeneration of shoots using three explants of two tomato varieties. A. Multiple shoot initiation on MS with 2.0 mg/l BAP + 0.5 mg/l IAA in case of BARI tomato-15 from cotyledonary leaf explants; B. Development of multiple shoots on same media and same variety mention as Fig A; C Multiple shoot formation from hypocotyls explants on MS with 2.0 mg/l BAP and 0.2 mg/l IAA in BARI tomato-9; D. Elongation of multiple shoots from hypocotyls explants of BARI tomato-15 on same media mention as Fig C; E. Formation of multiple shoots from cotyledonary node explants on MS with 2.0 mg/l BAP and 0.2 mg/l IAA in BARI tomato-9; F. Development of multiple shoots from cotyledonary node explants on MS with 2.0 mg/l BAP and 1.0 mg/l IAA in BARI tomato-15; G. Initiation of multiple shoots from hypocotyls explants on MS with 2.0 mg/l BAP in BARI tomato-15; H. Well developed shoots from hypocotyls explants on same media mention as Fig G; I. Shoot formation from hypocotyl explants on MS with 2.0 mg/l BAP + 0.5 mg/l Kn + 1 mg/l IAA in case of BARI tomato-15.; J. Formation of roots on same media and same variety mention as Fig I; K. Root formation on half strength of MS media without any hormonal supplements; L.Proper acclimatization of BARI tomato-9 in plastic pot.

was also noticed that, roots were initiated from the shoots on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l Kn + 1.0 mg/l IAA (Fig. 3J), which support the observation made by Shyluk and Constabel (1976).

(Fig. 3 K). Though Devi *et al.* (2008) reported that the best rooting was found to be in half-strength medium supplemented with 0.2 mg/l IBA. Bhushan and Gupta (2010)

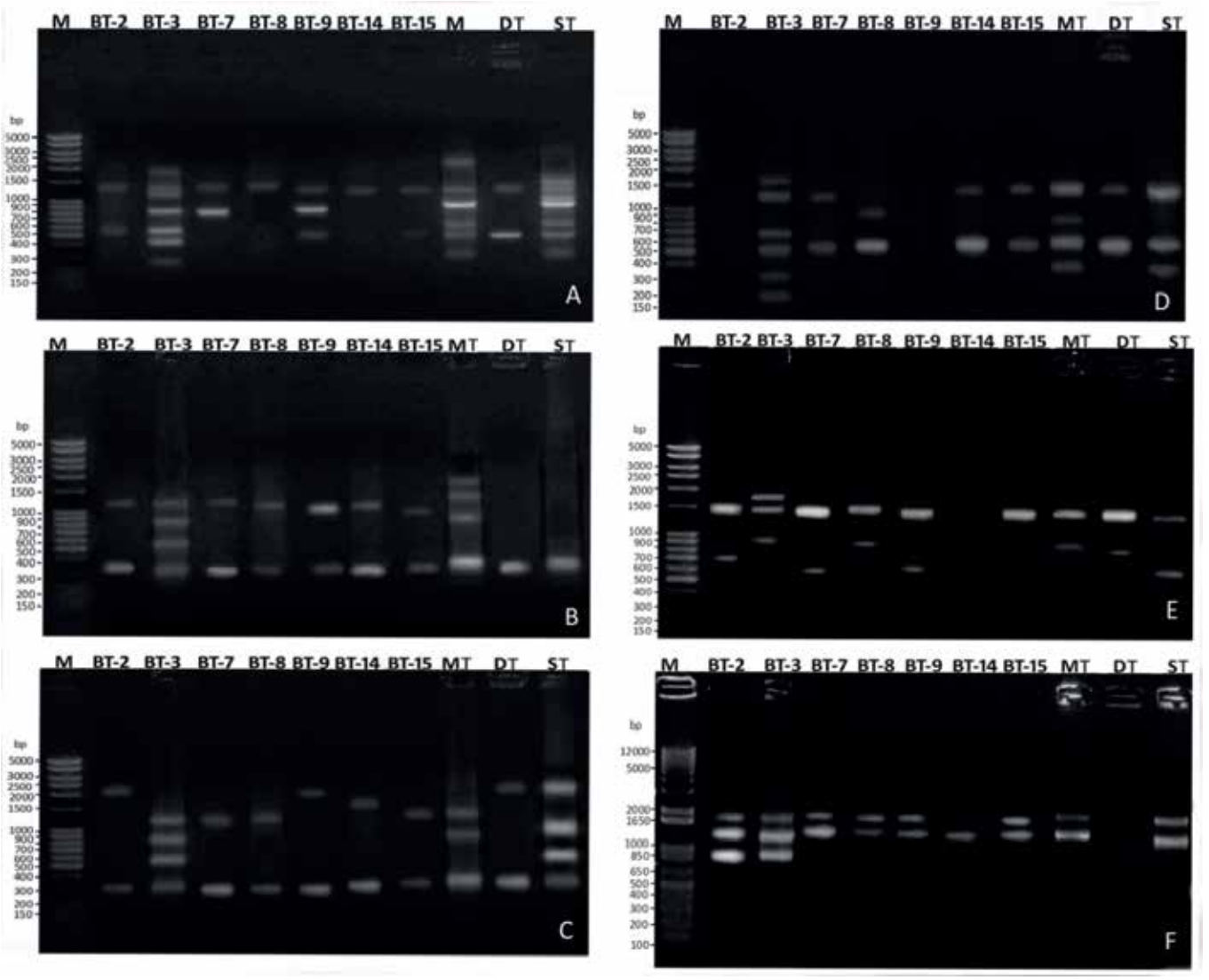


Fig. 4. RAPD analysis with different primers. (A) OPA-1 (5'-CAG GCC CTT C-3'); (B) OPA-4(5'-AAT CGG GCT G-3'); (C) OPA-5 (5'-AGG GGT CTT G-3'); (D) OPA-10 (5'-GTG ATC GCA G-3'); (E) primer-6 (5'-CCT GGG CTT A-3'); (F). primer-12 (5'-GTA TGG GGC T-3').

M=1 Kb DNA ladder, BT-2=BARI tomato-2, BT-3=BARI tomato-3, BT-7=BARI tomato-7, BT-8=BARI tomato-8, BT-9=BARI tomato-9, BT-14=BARI tomato-14, BT-15=BARI tomato-15, MT=Mintoo tomato, DT=Delta tomato and ST=Sawsan tomato.

Full and half strength of MS medium was tried for the formation of roots. Well developed and healthy roots were observed on half strength MS medium for both the varieties

and Velcheva *et al.* (2005) also reported formation of roots in hormone free MS medium. Plantlets of BARI tomato-9 and BARI tomato-15 variety were transferred into small plastic pots for proper acclimatization (Fig. 3L).

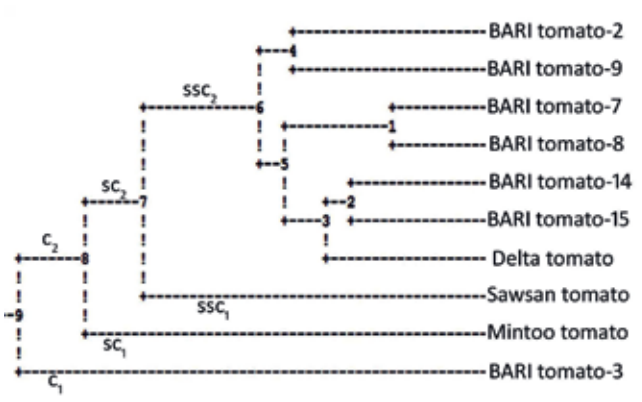


Fig. 5. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among 10varieties of tomato (Lycopersicon esculentum. Mill) by RAPD analysis.

In a separate set of experiment, six primer combinations, namely, OPA-1, OPA-4, OPA-5, OPA-10, Primer-6, and Primer-12 were used for RAPD analysis of 10 tomato varieties. Each primer combination showed different banding patterns. The 10 tomato varieties selected for the present study represent a broad spectrum of variation for several phenotypic traits and in their provenance. Only 3 common bands of different sizes were observed in three primer combinations (OPA-1, OPA-2 and OPA-5) (Fig. 4). The different sized common band indicated the sharing of similar DNA fragments among 10 varieties. Alam *et al.* (2012), was found two fragments of 1000 bp and 700 bp were common in the three tomato varieties (BARI tomato-2, BARI tomato-3 and BARI tomato-11) in Bangladesh in which the present study common fragment was 1200 bp and 450 bp. Afroz *et al.* (2013) found common band with primer OPA-1 in three morphological forms of *Alocasia fornicate* (Kunth) Schott.

These results indicated that the sequences of CAG GCC CTT C (OPA-1) are available in different species. Although these 10 varieties had some common RAPD bands, sufficient polymorphisms regarding RAPD fragments were observed. The primer sequence, band size and banding pattern of 10 tomato varieties were shown in Table-II The six primers generated 140 distinct bands of which 110 were considered as polymorphic. An average of 23.3 countable bands and18.3 polymorphic RAPD bands generated per primer showing 80.70% polymorphisms which indicated the high level of polymorphisms. Band size ranging from 200 - 2400 bp of PCR amplification products scored for all primers. Among the six primers OPA-2 and OPA-5 produced highest number of polymorphic bands. In contrast, the primer OPA-1 and OPA-5 generated the least number of polymorphic bands. A diverse level of polymorphism in different crops have been reported such as Chickpea 98.14% (Rasool 2013), Brassica 98.03% (Ghosh *et al.* 2009) and Chilli 90% (Paran *et al.* 1998). Wide range of polymorphism in tomato varieties was reported earlier using RAPD markers. Alam *et al*. (2012), was reported 94.168% polymorphism on BARI tomato varieties of Bangladesh. Naz *et al.* (2013) scored a high degree of polymorphism in 25 tomato cultivars using RAPD markers. The molecular weight of bands analyzed by them ranged from 400 and 2500 bp of which 72.60% were polymorphic.

The values of pair-wise comparison Nei's (1972) genetic distance among 10 tomato varieties computed from combined data from the six RAPD primer ranged from 0.1035 - 0.6769 (Table-III). The highest genetic distance (0.6769) was found between BARI tomato-3 vs Mintoo tomato. The lowest (0.1035) genetic distance was observed between BARI tomato-7 and BARI tomato-8. The difference between the highest and the lowest value of genetic distance revealed the

**Table II. Compilation of RAPD analysis in 10 varieties of tomato (*Lycopersicon esculentum* Mill.)**

| Primer codes | Sequences (5'—3') | Total bands | Size ranges (bp) | Number of Polymorphic bands | Polymorphisms (%) |
|---|---|---|---|---|---|
| OPA-1 | CAG GCC CTT C | 33 | 300-2400 | 23 | 70 |
| OPA-4 | AAT CGG GCT G | 22 | 450-1900 | 12 | 54.54 |
| OPA-5 | AGG GGT CTT G | 25 | 450-2400 | 15 | 60 |
| OPA-10 | GTG ATC GCA G | 23 | 200-1600 | 23 | 100 |
| Primer-6 | CCT GGG CTT A | 18 | 600-1750 | 18 | 100 |
| Primer-12 | GTA TGG GGC T | 19 | 600-1850 | 19 | 100 |
| Grand total | | 140 | | 110 | 80.7 |

**Table III. Summary of Nei's (1972) genetic distances of 10 varieties of (*Lycopersicon esculentum* Mill.)**

| Varieties | BT-2 | BT-3 | BT-7 | BT-8 | BT-9 | BT-14 | BT-15 | MT | DT | ST |
|---|---|---|---|---|---|---|---|---|---|---|
| BT-2 | **** | | | | | | | | | |
| BT-3 | 0.3042 | **** | | | | | | | | |
| BT-7 | 0.1989 | 0.3267 | **** | | | | | | | |
| BT-8 | 0.2397 | 0.3267 | 0.1035 | **** | | | | | | |
| BT-9 | 0.1790 | 0.3973 | 0.1989 | 0.2397 | **** | | | | | |
| BT-14 | 0.1989 | 0.3732 | 0.1790 | 0.1406 | 0.1989 | **** | | | | |
| BT-15 | 0.1989 | 0.3732 | 0.2191 | 0.2191 | 0.1596 | 0.1406 | **** | | | |
| MT | 0.4473 | 0.6769 | 0.4220 | 0.4220 | 0.3973 | 0.3267 | 0.2822 | **** | | |
| DT | 0.2397 | 0.4220 | 0.2191 | 0.2191 | 0.2397 | 0.1406 | 0.1790 | 0.2822 | **** | |
| ST | 0.3973 | 0.6144 | 0.3267 | 0.3732 | 0.3497 | 0.2822 | 0.2822 | 0.3497 | 0.1989 | **** |

wide range of variability persisting among the 10 tomato varieties. High genetic distance values between varieties pair were found due to difference in genetic constituent. The varieties of lowest genetic distance can be used as parental source for breeding line to improve tomato varieties. Tabassum *et al*. (2013) reported the values of pair-wise genetic distance ranged from 0.1838 - 0.9049 in tomato variety of Bangladesh. Sharifova *et al.* (2013) had found the genetic similarity among evaluated genotypes ranged from 0.188 - 1.000 on 19 Azerbaijan tomatoes. The lowest genetic distance was found between BARI tomato-7 vs BARI tomato-8. This result will be useful for designing future breeding programs.

## Conclusion

From the results obtained in the present investigation it can be concluded that this regeneration protocol is simple, reproducible and genotype independent. This optimized regeneration protocol can be efficiently used for *Agrobacterium* mediated genetic transformation in tomato. To characterize the tomato variety of Bangladesh using PCR based RAPD primers, this information would be helpful for future breeding program as well as patenting each variety to prevent varietal piracy.

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