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Embryo rescue of inter-specific hybrid of *Solanum lycopersicum* × *S. neorickii*

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Abstract

The tomato wild accession LA2325 (*Solanum neorickkii*) shows strong resistance to early blight (EB) caused by *Alternaria solani*. To study genetics of the resistance and to transfer the resistance to cultivated tomato, crosses were made between accessionLA2325 and highly susceptible genotype Hawaii 3998. The crosses yielded no viable seeds. It is well known that fertilization barriers like incompatibility and embryo abortion come in the way of development of inter-specific hybrid. This hampers introgression of resistance to cultivated tomato. In the subsequent season, pollen from LA2325was used to pollinate the susceptible cultivar and pro-embryos were collected from developing fruits 39-49 days after pollination. Rescue of immature embryos was done on Murashige and Skoog medium (MS-medium). Pro-embryos from fruits of 42 - 44 days after pollination gave good response. A total of ten plants were regenerated. Developed seedlings were hardened and shifted to green house. The hybridity of the rescued plants was confirmed by comparing the morphological characters of the interspecific hybrid and parents. Further, cleaved amplified polymorphic sequences (CAPS) marker was also used for verifying the hybridity. The embryo rescued inter-specific hybrid is being used as a resource for generating different genetic populations to study the genetics of the EB resistance and to transfer the resistance to cultivated tomato.

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Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop grown globally. It is the highest produced vegetable at the global level in 2020 with an annual production close to 186MTthat constitutes 16 percent of total vegetable production (FAOSTAT 2021). India is the second highest producer of tomato (20.57 million tons in 0.81 million ha) in the world (NAAS 2022). Along with onions and potato, tomato is an important vegetable crop due to its culinary importance in India. Tomatoes are mostly consumed fresh and hardly 1% of the tomatoes produced in India are processed into value-added products like sauce, ketchup, puree, paste, soup, pickled on a commercial scale (Subramanian 2016). There is a need for consistent tomato production and supply due to the crop's wide range of applications and significance. However, biotic stresses including viruses, fungi, insects, nematodes, etc. and different abiotic stresses like high temperature, salinity, and flooding are hampering the commercial production of tomatoes. Among different fungal diseases early blight caused by several species of Alternaria including *Alternaria linariae* (which includes *A. solani* and *A. tomatophila*), as well as *A. alternata* is highly destructive in many tomato producing areas worldwide (Sherf and MacNab 1986).Tomato fruit yield loss up to 79% is reported due to early blight in major tomato growing countries like Canada (Basu1974), India (Datar and Mayee 1982), USA (Sherf and MacNab 1986), Nigeria (Gwary and Nahunnaro 1998),Australia, Israel and UK (Vloutoglou and Kalogerakis2000).

Due to lack of high level of resistance in tomato cultivars, tomato growers have adopted disease management strategies such as cultural practices and fungicide applications. The sources of resistance have been reported in wild species of tomato especially in *Solanum habrochaites, Solanum arcanumand S. pimpinellifolium* (Ashrafi and Foolad 2015, Chaerani and Voorrips 2006, Chaerani et al. 2007). Several quantitative trait loci (QTL)with low individual QTL effects were detected in populations derived from these wild species. Further, complex inheritance of early blight resistance and association with undesirable horticultural characters hampered the resistance breeding programme in tomato (Adhikari et al. 2017). It necessitated search for new sources of early blight resistance. Of late, three genotypes including two tomato wild species LA2325 (*S. neorickii*) and WIR3928 (*S. cheesmaniae*), and an advanced breeding line H-88-78-1 showed immune reaction to *A. solani* at four weeks after sowing (Yerasu et al. 2019). Introgression of the resistance into cultivated tomato needs development of interspecific hybrids. Owing to incompatibility barriers it is not possible to develop interspecific F1 hybrids by conventional methods. In such cases the technique of embryo reuse was employed in tomato (Barbono and Topoleski 1984, Pico et al. 2002, Bhattarai et al. 2009, Encina et al. 2012, Kharkongar et al. 2013, Sorab et al. 2015). In this paper, we present the successful embryo rescue of interspecific hybrid carried out to overcome fertilization barriers between *A. solani* resistant LA2325 (*S. neorickii*) and highly susceptible cultivated tomato (*S. lycopersicum*) line Hawaii-3998 (Yerasu et al. 2019).

Materials and Methods

Plant material and crossing technique: Genotypes used in these experiments included *S. neorickii* accession LA2325introduced from C.M. Rick, Tomato Genetics Resource Center, University of California, Davis. Early blight susceptible cultivated tomato (*S. lycopersicum*) line Hawaii-3998. The accession LA2325 was used as pollen parent and Hawaii-3998wasused as female parent. The seeds of both the parents were germinated in pro-trays having a mixture of manure, soil and sand (1:1:1ratio). Four weeks old seedlings were transplanted on raised beds in open field conditions. Crossing activities were performed during October and November months. We followed hand emasculation and pollination for making hybridization. The flowers of female parent Hawaii-3998 were hand emasculated at around 2–4 p.m. a day before pollination. Hand pollination was performed following day between 09:00 a.m and 11:00 a.m using fresh pollen grains collected from opened flowers of *S.neorickii*. The pollinated flowers were labeled properly indicating date of pollination.

Embryo culture: To overcome the inhibition of hybrid embryo development, the technique of embryo-rescue was employed. Fruits were harvested between 40 to 49 days after pollination and washed in tap water for 5 min. Then the fruits were disinfected in 1.5% sodium hypochlorite for 30 min and rinsed five times with sterile distilled water. After sterilization, the fruits were cut open aseptically. The gelatinous ovule coating was removed. Pro-embryos were collected and rescue of immature embryos was done on MS-medium (Murashige and Skoog 1962). The regenerated shoots were further sub-cultured on MS medium and allowed for shoot elongation. The elongated shoots were cut aseptically and further sub-cultured on half-MS medium for root formation. After proper rooting, plants were shifted in small cups with sterile cocopeat, vermiculite and perlite (1:1:1 ratio). The plants were recovered with perforated polythene bags for two weeks. The hardened plants were shifted to big containers with soil, farm yard manure with sand in green house and maintained till fruit formation. Hybridity of embryo rescued plants was confirmed by morphological observation and by molecular means also.

Marker assays to confirm hybridity: At molecular level, cleaved amplified polymorphic sequence (CAPS) marker C2_At3g06050(F: 5'-ATACACTATGAACGGTTGGGCAG-3' and R: 5'-AAACTCTTGTGGAAGCTTCCATC-3') located on chromosome 1 of tomato digested by restriction enzyme DdeI was used to confirm hybridity of embryo rescued F1 plants. For DNA extraction, young leaves collected in a 1.5 ml centrifuge tube were used by following a standard protocol (Prasanna et al. 2015). A 25 µL volume of PCR reaction consisting of 6.25 µL master mix (2.25 mM MgCl2, 0.2 mM dNTPs, 0.5 U Taq DNA

polymerase, 10× PCR buffer, and 0.4 µL primers) and 16.75 µL of nuclease-free water were used for the PCR. The PCR procedure employed for the markers involved 94 0C for 4 min, then 35 cycles of 94 0C for 1 min, 55 0C for 1 min, and 72 0C for 2 min, with a final extension of 5 min at 72 0C. The restriction enzyme DdeI was used to digest amplified PCR products of CAPS marker. DNA bands were identified using ethidium bromide staining after the PCR and restriction digested products were separated on a 2% agarose gel at 120 V for 60–70 minutes.

Results and Discussion

Early blight is a complex disease and effects all above ground parts of the plant and at all growth stages of tomato plant. It causes collar rot on young seedlings, early blight on foliage, stem lesions on adult plant and fruit rot on fruits (Walker 1952). Early blight is a destructive fungal disease causing considerable loss in tomato production world over. Presently, there is no relievable early blight resistance in cultivated tomatoes (Adhikari et al. 2017). Wild tomato species are valuable genetic sources to introgress resistance to diseases and to improve important agronomic traits (Esquinas Alcazar 1981, Laterrot 1989, Rick et al. 1987). Accordingly, tomato wild species contributed greatly to the modern cultivated tomatoes with respective to agronomic characters and resistance to different biotic and abiotic stresses. Since 1982, there have been one to two reports each year of resistances discovered in wild relatives of the tomato (Rick and Chetelat 1995) and more than 40 resistance genes have been introgressed from *Solanum peruvianum, S. cheesmanii, S. pennellii,* and several other wild relatives (Rick and Chetelat 1995,Reem and Toby 2007).

Tomato wild accession LA2325 (*S. neorickii*) showed immune reaction to collar rot caused by *A solani* (Yerasu et al. 2019). To understand the genetics of resistance and to transfer resistance to cultivated tomato, development of interspecific F1hybrid is an essential first step. The initial crosses made between Hawaii-3998 (as female parent) and LA2325 (male parent) were successful in setting fruits. However, we didn't find a quality seed when red ripen fruit was cut open. In tomato, both pre-zygotic and post-zygotic interspecific crossing barriers are the main hindrances in development of interspecific hybrids and in further introgression of desirable characters into cultivated tomato from wild species (Barbono and Topoleski 1984). In tomato, pollen viability and fruit set are highly influenced by different whether parameters like day/night temperature and humidity. In the present study, there was a good fruit setting. In total, 54 fruits were dissected for isolation of immature embryos from fruits collected after 40-49 days after pollination. Brown necrotic deformed pro-embryos were observed in dissected seeds from fruits of 47 days postpollination. This indicated post-fertilization abnormalities leading to disintegration of embryos. Similar kind of observations was reported earlier also (Piosiket al. 2019, Sohrab et al. 2015). The ovules collected from fruits set42-44 days post pollination were sound and gave large number of pro-embryos that can be cultured in vitro. The pro-embryos from fruits set 43 days after pollination gave more embryo rescued plants (Table1).

Table1: Number of fruits cut, seeds dissected, embryos on media and number of plants grown from the inter specific cross of *S. lycopersicum* (Hawaii-3998) and *S.neorickii* (LA2325)

Days after pollination	No of fruits cut	No. of seeds dissected	No. of embryos on media	No. of plants grown
40	4	18	4	nil
41	4	17	nil	-
42	10	49	5	1
43	14	78	23	6
44	6	40	14	3
45	10	12	nil	-
46	2	22	nil	-
49	4	26	nil	-
Total	54	262	46	10

Embryo rescue is one of the techniques to develop interspecific hybrids. Other techniques include use of gamma-ray irradiated pollen grains for pollination, use of polyploidy bridge crossing (Poysa 1990) and ovule culture (Imanishi et al. 1985).

Composition of media on which pro-embryos are rescued plays a greater role in the success of embryo rescue. Various researchers have reported different media combinations used in successful regeneration of intercross pro-embryos (Hossain et al. 2003, Bhattarai et al. 2009, Kharkongar et al. 2013, Sohrab et al. 2015). In our study, the MS medium without any addition of phyto-hormones successfully supported the pro-embryos germination and shoot growth. Half-MS medium without any addition of phyto-hormones successfully supported root formation (Figure 1).

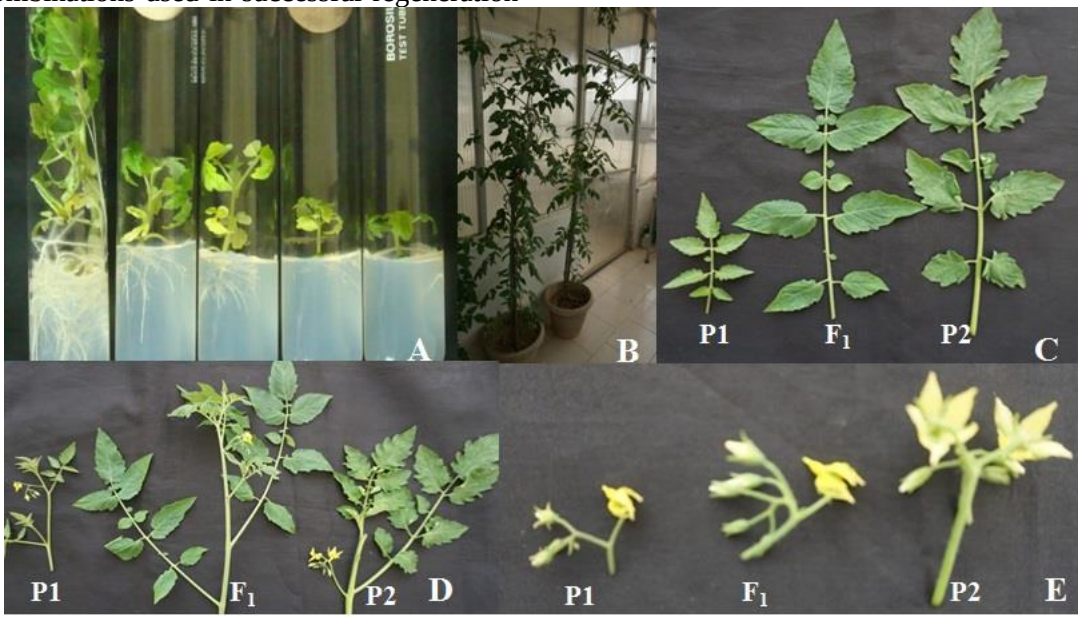

Figure1: A− Embryo rescued interspecific hybrids on MS-medium; B− Fully grown embryo rescued interspecific hybrids in green house; C−, D− and E− Leaf, twigs and flowers, respectively of LA2325 (P1), interspecific hybrid (F1) and Hawaii-3998 (P2).

Further, half MS medium was used for maintaining the embryo rescued plants in vitro through cuttings. DNA was isolated from the leaves collected from embryo rescued plants. Marker assay of parental and interspecific F1s with the CAPS marker (C2_At3g06050+DdeI) established the hybridity of the entire ten embryo rescued interspecific F1 hybrids (Figure 2).

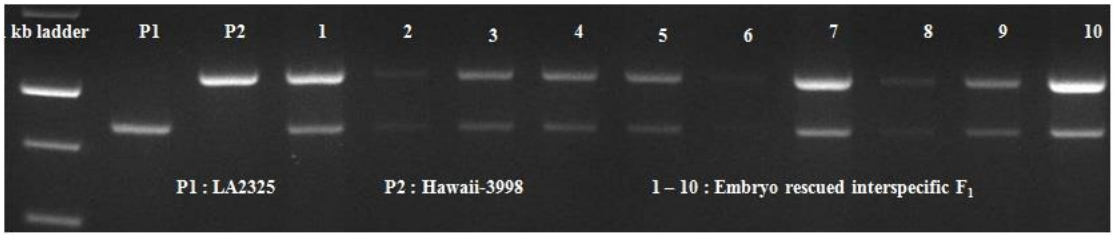

Figure 2: Molecular confirmation of hybridity of embryo rescued interspecific hybrids with CAPS marker (C2_At3g06050+*DdeI*)

Among all, seedlings from the cutting of one interspecific F1hybrids were hardened and grown in greenhouse. Further, morphological observations also confirmed hybridity (Figure 1).

S. neorickii is a green fruited self-compatible species of tomato with a large geographical range that extends from near Paute in central Ecuador to the Cusco area in southern Peru (Baek et al. 2015). Successful interspecific hybridization between cultivated tomato and *S.neorickii* accession were reported (Rick 1979). *S. neorickii* has large geographical range and accordingly has large variation (Baek et al. 2015). In the present study, interspecific cross between Hawaii-3998 and LA2325 was not successful. Embryo rescue of pro-embryos42-44 days after pollination on plain MS medium indicated on set of post-zygotic interspecific barriers in the present interspecific cross. By 42-44 days, embryo might have developed properly and lack of sufficient endospermic resources thereafter may be the reason for improper development of seeds naturally. Once the excised 42-44 days embryos were kept on plain MS medium, they were able to germinate in vitro. The developed interspecific hybrid can be useful resources to develop filial and backcross generations for the study of genetics and simultaneous transfer of collar rot (*A solani*) resistance in the genetic background of cultivated tomato.

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सारांश

टमाटर की जंगली प्रजाति एलए–2325 (*सोलनम नीओरिक्की*) अगेती झुलसा (*अल्टरनेरिया सोलानी*) के विरुद्ध बहुत अधिक प्रतिरोधी है। प्रतिरोध की आनुवंशिकी का अध्ययन एवं सामान्य टमाटर में रोग प्रतिरोध क्षमता को स्थानांतरित करने के लिए इस प्रतिरोधक जंगली प्रजाती को अत्यधिक अतिसंवेदनशील टमाटर हवाई 3998 के साथ संकरण किया गया, जिससे कि कोई जिंदाबीज नहीं प्राप्त हुआ। यह सर्वविदित है कि अंतर–विशिष्ट संकर के विकास में निषेचन अवरोध जैसे असंगति और भ्रूण गर्भपात जैसे बाधाएं आते हैं, जिसकी वजह से सामान्य टमाटर में रोग प्रतिरोध का स्थानांतरण बाधित होता है। इसका निवारण करने के लिए, अगले मौसम में एलए–2325 के पराग को हवाई 3998 के साथ परागित किया। परागण के 39–49 दिनों के बाद विकासशील टमाटर के फलों से प्रो–भ्रूण एकत्र किए गए। इन अपरिपक्व भ्रूणों को एमएस – माध्यम पर बचाव किया गया। अंत में यह पाया गया कि परागण के 42–44 दिनों के विकसित फल से प्रो–भ्रूणों ने अच्छी प्रतिक्रियादी। कुल दस पौधों काटि सूकल्चर में पुनर्जनन किया गया और फिर सूक्ष्म–पौधों का हार्डेनिंग करके ग्रीनहाउस में स्थानांतरित किया गया। पुनर्जीवित किये गए पौधों की संकरता की पुष्टि संकर पौधों और जनकों के आकारिकी लक्षणों की तुलना के आधार पर की गई। इसके अलावा पौधों की संकरता का सत्यापन क्लीव्ड एम्प्लीफाइड पॉलीमॉर्फिक सीक्वेंस मार्कर के द्वारा भी किया गया। अंततः, भ्रूण बचाव द्वारा अंतर–विशिष्ट संकर टमाटर का उपयोग अगेती झुलसा प्रतिरोध के आनुवंशिकी का अध्ययन एवं सामान्य टमाटर में प्रतिरोध को स्थानांतरित करने के लिए एक संसाधन के रूप में किया जा रहा है।

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