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# Scope and Utility of Micro-morphological Markers in Hybridity Testing of Tomato (*Solanum lycopersicum* L.)

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**ABSTRACT:** Conventionally, grow-out-test, the only official method of genetic purity testing, is season dependent, labour intensive; and locks huge investment. Alternatives needed to be developed to test the genetic purity of a given seed lot in a shortest possible time. To bridge the above gap, the present study was undertaken to identify the four hybrids and their parental lines (six) in tomato using micro-morphological markers at seedling stage. Among the various micro-morphological characters: anthocyanin pigmentation pattern on midrib of cotyledonary leaf, shape of cotyledonary leaf in cross section, shape of first leaf blade, blistering pattern and positioning of veins on the lamina of first leaf, identified as new characters; reported as first of its kind in hybridity testing of tomato, which are easy and cost-effective. The shape of cotyledon and anthocyanin pigmentation on lower surface of first leaf were also identified as first of their kind in tomato for parental lines identification.

**Keywords:** Hybridity testing, Micro morphological markers, Tomato

One of the avenues given for improving the productivity is developing and adopting hybrid tomato (*Solanum lycopersicum* L.) cultivation. Hybrids combine good characters from both the parental lines and have an advantage over their parental lines or any other open pollinated varieties. Since, the inception of very first tomato hybrid “Single Cross” in 1946 [1], many hybrids both from public and private sector are flooding the seed markets meeting the present and future criteria of producers and consumers. In the process of surviving and making profits, seed companies develop new cultivars continuously with added value. The average turnover time of commercial tomato cultivars is approximately five years. Thus, the companies are involved in a very competitive breeding practice and try to recover the investment costs by selling their seeds at higher prices. The released new hybrids tend to get acceptance only when they are high yielding, accompanied by genetic purity and uniformity. Seeds of such cultivars fetch a very high price in the market. The innate potential of hybrid seed draws a flak in the market when the same hybrid is sold in different names or spurious seed is sold in the name of a well performing hybrid. Thus, to upkeep the interest of farmers and for getting true value to the seed they are procuring, certain measures are put in practice. Among them, genetic purity testing was made mandatory for any certified hybrid seed offered for sale in India.

Grow-out-test (GOT) is the conventional method for testing the genetic purity of hybrid seed lots; comparing test sample plants with authentic sample for phenotypic characters throughout the growing season. Until date, morphological characters both qualitative and quantitative form the bulk of varietal characterization and they have been universally accepted, as undisputed characters when used in sequential fashion thus making them convenient for distinguishing the different varieties. But, many of these characters are multigenic or quantitative, thus their expression is altered by the environment and also, the time taken to observe the characters over a growing season has generated interest in utilizing alternate but precise methods for varietal identification.

Citing the requirement of a reliable marker system which will be handy, stable with ease of conducting the test, there is an urgent need to develop precise and efficient method to ensure the genetic purity of hybrid seeds and be made available for seed marketing well in time. Micro-morphological markers may be considered as a ray of hope apart from the molecular markers which are fast, reliable but costly. Micro-morphological markers can be studied in seed and early stages of seedling like trichomes, stomata, anthocyanin pigmentation, root characters, cotyledon characters and first leaf characters, which help to ascertain the cultivars without incurring much cost. The potential advantage of exploring the micro-morphological characters in the present study will be the simplicity in examining, low-cost, by-passing requirement of skilled personnel and sophisticated facilities. The present study was conducted to assess the feasibility of new characters which can distinguish the hybrid from its parental lines during the early stages of seedling development.

## MATERIALS AND METHODS

A total of four tomato hybrids and their parental lines (Table 1) developed and released by Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi were used in the present study.

**Table 1.** Tomato hybrids and their parental lines used in the study

| Hybrid | Female | Male |
|---|---|---|
| PH-1 | Female-1 | Male-1 |
| PH-2 | Female-2 | Male-2 |
| PH-4 | Female-2 | Male-1 |
| PH-8 | Female-3 | Male-3 |

An array of micro-morphological characters of cotyledon, hypocotyl, first leaf and roots were studied at early stages (seedling emergence to three weeks) of seedling growth to distinguish the genotypes for their possible variability. In cotyledon, orientation with respect to stem apex, cotyledon apex shape, base of cotyledon, colour at senescence, anthocyanin pigmentation pattern and cotyledon shape in cross section were studied. With respect to the first leaf, its orientation, shape of leaf blade apex, shape of leaf blade base, venation and leaf lamina features, leaf blade shape and anthocyanin colour distribution (under hydroponics) were studied.

### Studies on cotyledonary leaf and first leaf lamina features under polyhouse conditions

These studies were conducted at Centre for Protected Cultivation and Technology, ICAR-IARI, New Delhi. For raising the seedlings under polyhouse conditions, seedlings were raised in plug trays using coir pith and vermiculite as base. Single seedling was maintained in each plug of the tray and watering was done on every alternate day until the date of recording observations. The seedlings were raised up to 15 – 18 days and observations were recorded on fully expanded cotyledonary leaves which coincides with the appearance of leaf primordial.

Cotyledon characters like orientation with respect to stem apex, cotyledon features at base of cotyledon, colour at senescence were observed from the day of emergence to three leaf stage in the genotypes under study. Also, shape of cotyledonary leaf under cross section was observed in fully expanded cotyledon leaf was selected from seedling which had primary leaf primordial, just visible to the naked eye. Each cotyledonary leaf was used to obtain the cross section from the middle portion of its leaf using a potato tuber as a base. Sections were taken in transverse plane manually and specimens were viewed under Leica D750 stereo microscope at 4X magnification and photographs were taken using Nikon camera (DFC 295), an attachment with the microscope.

Characters like leaf blade shape, leaf blade apex and base were observed for their variations during 8 – 21 days from emergence. Observations for leaf lamina like blistering and vein position were observed during 8-11 days from sowing using Leica florescence stereo zoom microscope (Model M216 FA).

### Studies on lamina shape and anthocyanin pigmentation pattern under hydroponic conditions

Seedlings were raised under hydroponics under glasshouse conditions (16° to 22°C) of National Phytotron facility, ICAR-IARI, New Delhi. Medium used was Hoagland's solution. Seeds of all genotypes were first germinated in a petri-dish by placing them on two layers of blotter paper. Four days old germinated seedlings were transferred into the hydroponics medium.

For studying the anthocyanin pigmentation pattern on cotyledonary leaf, initially, one part of Hoagland's solution was mixed with 3 parts of water and used as a medium for growing the seedlings for 2 days until the seedlings were established. Later, 2 parts of Hoagland's solution mixed with 2 parts of water and used as medium under hydroponics until 21 days. Low concentrations of Hoagland's solution used as the medium, led to differential expression of anthocyanin pigmentation on the cotyledons (10 to 12 days old) and first leaf in all genotypes (16 to 18 days old).

For studying the lamina shape of first leaf, hydroponic cultures were raised as per the procedure explained above except that Hoagland's solution without dilution (with water) was used as medium. Changes in leaf shape was observed from 12 to 21 days and the distinct differences observed from 15 to 18 days.

## RESULTS AND DISCUSSION

Quality seed is the most vital and basic input for any successful crop production program. Parameters such as seed viability, seedling vigour and health status are relatively easy and quickly determined; genetic purity testing often poses challenge as most of the traits that define a hybrid or variety are not easily discernible at an early stage of seedling development and genetic purity testing has been made mandatory for certified seed produced employing chemical hybridizing agents or manual emasculation and pollination. Hence, the certified hybrid seed produced cannot be sold immediately in the ensuing season as their genetic purity certification requires a time-taking grow-out-test, the only approved practice as on date, which might last several months. Thus the certified hybrid seed produced is held back until the results are declared. As grow-out-test is resource demanding, labour intensive and time-consuming; necessitated new innovative and efficient methods of genetic purity testing to be devised.

Any test on genetic purity that can be conducted to assess the genetic purity within 30 days for a given crop will be a huge advantage having said about the disadvantages of traditional grow pout test. The block method followed in commercial seed production program in tomato, where male and female parental lines are raised in separate blocks minimizes the chance of male seed contamination. Hence, emphasis was placed on distinguishing the female parental line and the hybrid. Identification of such morphological markers will be more useful and realistic in terms of determining the hybrid seed purity, as these were easier and cost-effective.

### Cotyledon characters

Observations were recorded continuously from the day of seedling emergence to three leaf stage for various traits among tomato hybrids and their parental lines. No differences were observed, among the hybrids and parental lines, for qualitative characters like orientation of cotyledonary leaf with respect to stem apex, base of cotyledon, colour of cotyledonary leaf at senescence stage. Shape of the cotyledon was useful in differentiating Male-2, the male parental line of PH-2 hybrid. Male-2 has a unique ellipsoid cotyledon with round tip rather than the lanceolate shape and pointed cotyledons common to the rest of the genotypes (Figure 1).

With respect to shape of cotyledonary leaf in cross section, distinct differences were observed for shape of cotyledonary leaf in cross section among the genotypes, under study. The differences in the textural surface of cotyledonary leaf as depicted by the features in cross section proved to be useful in identifying the hybrids from their parental lines. Midrib was prominently seen in genotypes *viz.,* PH-1, PH-2, PH-4, PH-8, Female-1, Female-3, Male-1, Male-2 and Male-3 whereas it was less evident in Female-2. Thus, PH-2 and PH-4 could be identified from their corresponding female parental lines based on midrib feature in cross section of cotyledonary leaves. PH-1 and PH-8 could not be distinguished from both their female parental lines, as both the hybrids and the female parental lines had distinct mid rib in the cross section of cotyledonary leaf (Figure 2). The prominent midrib feature found in the parental lines was always found in the hybrids suggesting its dominance. Thus, if the only male parental line had it but not the female, it could be used to establish the hybridity. This technique could be easily adopted for screening, even with a moderately thick (50μ) hand section, depicting clearly the phenotype. This character was proved to be useful in distinguishing hybrids PH-2 and PH-4 from their female parental lines; as the female parental line had no conspicuous midrib, as it was observed in male parental


**Figure 1.** Cotyledon shape in tomato hybrids and their parental lines

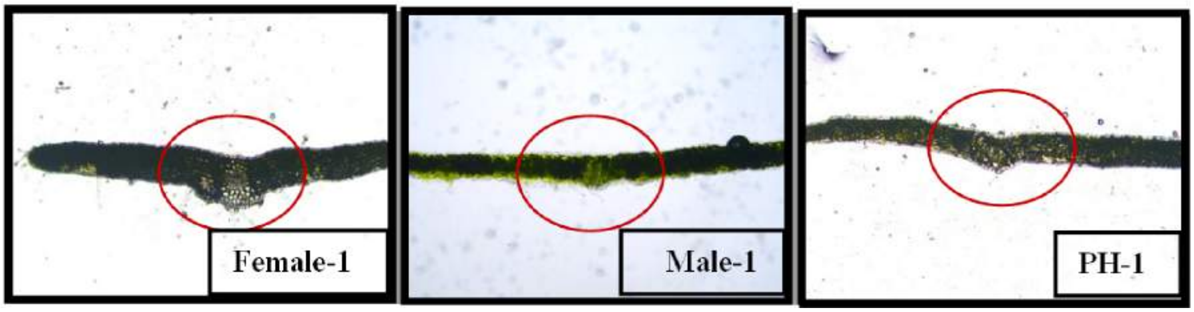


**Figure 2.** Shape of tomato cotyledonary leaf in cross section

line. Hwang and Conran [2] reported the usefulness of this character (shape of cotyledonary leaf in cross section) in distinguishing taxa of Casuarinaceae. This is the first report, where this trait had been depicted to be useful in identifying tomato hybridity.

### Anthocyanin pigmentation pattern on cotyledon and first leaf under hydroponics

Anthocyanins give typical characteristic colours like red, purple and blue. These can be synthesized under stress like exposure high temperature or sunlight [3] .They accumulate in leaves under different ontogenic stages associated with abiotic stress like ultra violet light, nutrient deficiency and biotic factors like herbivory and pathogen interaction [4]. It is a genetically controlled trait, but is highly influenced by the environment. In the present study, anthocyanin pigmentation on hypocotyl was uniform in all genotypes under study, making no distinction between hybrids and their parental lines. Styles *et al.* [5] reported that alleles of certain regulatory genes involved in anthocyanin production alter the pattern of production rather than the amount of pigmentation produced. This was best exemplified in R and B loci of maize, which govern a wide range of pigmentation patterns on diverse set of plant tissues.

Recessive gene *atv* is reported for expression of strong anthocyanin pigmentation in leaves and stems [6]. Genes like anthocyanin less (a), anthocyanin absent (aa), entirely anthocyanin-less (ae), anthocyanin free (af), anthocyanin gainer (ag), incomplete anthocyanin (ai), anthocyanin loser (al), anthocyanin reduced (are), without anthocyanin (aw), and baby lea syndrome (bls), negatively regulate the production of anthocyanins in vegetative tissues. Also, mutations like aw and bls were recorded for complete inhibition of anthocyanin biosynthesis [7].

For effective screening of anthocyanin pigmentation in DUS testing of sorghum, Anon [8] and Anon [9] proposed the evaluation of genotypes in sand or in the mixture of sand and compost by with-holding 'P' availability.

The present experiment was conducted to examine the pigmentation pattern under hydroponics, where low level of quantified nutrition was provided at sub-optimal temperature under glass house conditions. The methodology led to varied patterns of anthocyanin accumulation on midrib of cotyledonary leaf and lower surface of first leaf. Anthocyanin was observed along the midrib of cotyledonary leaf in Female-2, Female-3, Male-2 and PH-2, whereas no pigmentation was observed in rest of the genotypes. This differential expression in the genotypes enabled identification of the hybrids from their corresponding parental lines. PH-4 and PH-8 had no anthocyanin accumulation on midrib of first leaf following the male parental line character. The hybrids were distinct and could be identified from its female parental line, as the female had anthocyanin accumulation on midrib of cotyledonary leaf. Parental lines of PH-1 had no anthocyanin pigmentation, whereas parental lines of PH-2 had anthocyanin pigmentation on midrib of cotyledonary leaf making them difficult to identify the hybrids from their parental lines (Figure 3). In general presence of anthocyanin is a dominant trait. However, there were exceptions as reported in case of *Eluta* allele in antirrhinum and *cl-I* allele in maize. These alleles act as


**Figure 3.** Anthocyanin pigmentation on midrib of cotyledonary leaf

negative regulators of pigmentation (Dooner and Robbins, [10]). The absence of anthocyanin pigmentation in hybrids PH-4 and PH-8 suggests similar inhibitory gene action for this trait in tomato. A detailed genetic study of this trait is imperative before this trait is routinely used in hybrid purity testing of tomato.

Anthocyanin pigment production and distribution varied under hydroponics in glass house than those to polyhouse conditions. Anthocyanin distribution was seen distinctly on lower surface of first leaf for all studied genotypes except in Male-1 (male parental line of PH-1 and PH-4) making it distinct from others (Figure 4). Anthocyanin pigmentation on lower surface of first leaf could distinguish only male parental line (common to both hybrids) of PH-1 and PH-4. However, hybrids could not be distinctly identified from its parental line. This trait was identified as a unique marker for male parental line of PH-1 and PH-4 for its identification.


**Figure 4.** Anthocyanin pigmentation pattern on lower surface of first leaf

**Features of first leaf lamina under polyhouse conditions**

Characters like orientation of first leaf with respect to main axis, leaf blade apex shape, leaf blade base shape, leaf lamina shape, leaf lamina blistering and vein positioning were studied to explore possible variation in distinguishing the hybrids from their parental lines. Considerable and conspicuous variation was observed for primary leaf characters like leaf lamina shape, leaf lamina blistering and vein positioning in relation to lamina surface among the genotypes studied.

Leaf lamina texture, viewed under Leica florescence stereo zoom microscope (Model M216 FA) at 4X showed blistering pattern in some genotypes with deep seated veins (Figure 5). The character was clearly visible from 8-11 days from seedling emergence. In the female parental line of PH-2, the leaf lamina was flat and the veins were not conspicuously demarcated from its surrounding lamina tissue, whereas in the male parental line of PH-2, blistered texture could be observed on the lamina with veins having a clear demarcation from the lamina tissue. The similar pattern was observed in hybrid PH-2, distinguishing it from the female parental lines. Similar distinction was seen in female parental lines of PH-1 and PH-4 with flat leaf lamina, whereas male parental lines and hybrids had blistered leaf lamina. In PH-8, the leaf lamina texture was seen in both the parental lines making the hybrid also appearing similar.

Shape of leaf lamina on 18-21 days from seedling emergence was one more character, which proved to be

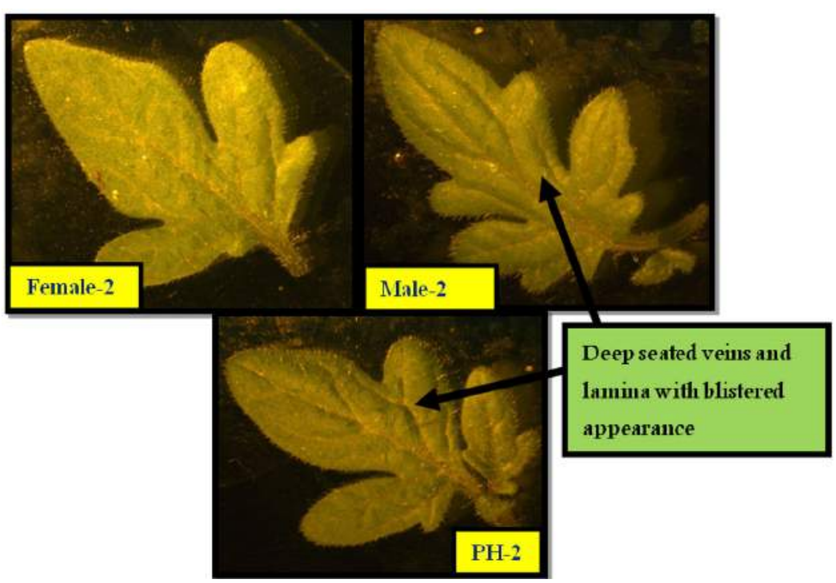


**Figure 5.** First leaf lamina under microscope in tomato hybrid PH-2 and its parental lines

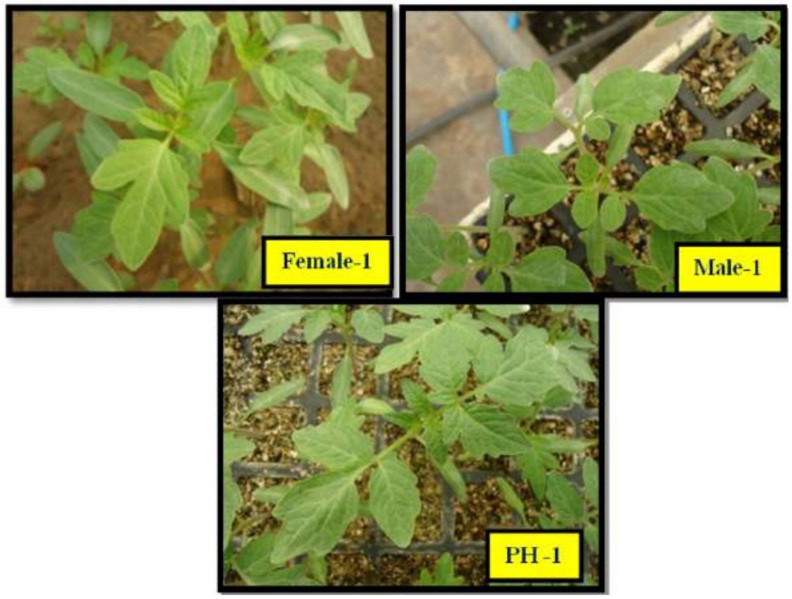


**Figure 6.** First leaf lamina shape on 18-21 days from seedling emergence under polyhouse conditions in tomato hybrid PH-1 and its parental lines

useful in identifying the hybrids from their parental lines in PH-1 and PH-4 (Figure 6). The leaf lamina of terminal leaflet in male parental line and hybrids of PH-1 and PH-4 had very shallow lobes, whereas the female parental line terminal leaflet in primary leaf had clear and deep lobes. The character did not qualify to distinguish the hybrids PH-2 and PH-8 from their parental lines.

### First leaf lamina shape under hydroponics

Leaf lamina shape has been one of the characters widely used in assessing genetic purity testing in crops like cotton, okra *etc.* But, this trait is highly dynamic and also dependent on age of the plant. The present study concentrated the changes in leaf lamina shape of first leaf under different environmental conditions with respect to hybrids and parental lines; and their possible utility in identifying the hybrids from its parental lines, particularly the female parental line. The leaf shape study focused two variables – one being placement in heteroblastic series and second one the age technically referred as ontogeny. The shape of tomato leaves differs from first leaf to second leaf. The shape of first leaf had different dimensions at different stages of its development. The leaf lamina shape at 18–21 days proved to be useful in identifying hybrids PH-1 and PH-4 from their female parental lines under polyhouse conditions.

Leaf lamina shape depicted variations at different stages of seedling growth in the genotypes, when tested under hydroponics (glass house conditions) of National Phytotron Facility in ICAR-IARI, New Delhi.

No variation was observed for PH-1 and PH-8 for leaf lamina shape, from the day of transfer to 21 days under hydroponics, with respect to their female and male parental lines. The lamina shape was seen as deeply lobed in PH-2 and its male parental line on 14–16 days from transfer. During the same period, the female parental line exhibited shallow lobed leaf lamina (Figure 7). In case of PH-4, the differences in leaf lamina shape were seen during 17-19 days from transfer into hydroponics. PH-4 and its male parental line had shallow lobes on leaflet in first leaf, whereas the female parental line had deep lobes (Figure 7). On 21 days from transfer, the terminal leaflet of first leaf in Male-1 parental line depicted very minimal lobes, making it distinct from others studied (Figure 7). Bar and Ori [11] reported that plants change the leaf shape quickly in response to varied environmental stimuli. The leaf shape tends to elongate under low light intensity. Chitwood *et al.*[12] correlated leaf size and serration with temperature and precipitation. First leaf lamina shape proved to be a useful trait to distinguish PH-2 from its female parental line under hydroponics on 15 day from its transfer into medium, whereas on 18 day from its transfer, PH-4 could be distinguished from its female parental line. This was another first report documented the utility of seedling leaf character for establishing hybridity.


PH-2 and its parental lines

**Figure 7.** First leaf lamina shape on 15 days from transfer into hydoponics in tomatohybrid

## CONCLUSION

The present study was aimed at identifying different morphological and molecular markers for assessing the genetic purity of hybrid seed to save the precious seed from its lock-in-period until the result is declared by conventional grow-out-test. Among the various micro-morphological characters: shape of cotyledonary leaf in cross section, anthocyanin pigmentation pattern on midrib of cotyledonary leaf, shape of first leaf blade, blistering pattern and positioning of veins on the lamina of first leaf, identified as new characters; reported as first of its kind in hybridity testing of tomato; the shape of cotyledon and anthocyanin pigmentation on lower surface of first leaf, respectively were also identified as first of their kind in tomato for parental lines identification. Micro-morphological when used in combination, have the potential to distinguish all the four tomato hybrids and their respective parental lines during seed and seedling stages itself, and also to spot offtypes and/or selfed plant; as a fast track, cost-effective (labour saving) and easy alternative to conventional grow-out-test.

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