# Hematoxylin staining as a potential screening technique for aluminium tolerance in pea (*Pisum sativum* L.)

In India[1], of the 49 million hectares (mha) of acid soil, 25 mha has pH below 5.5. Considerable genetic variability for aluminium (Al) tolerance exists in pea seedlings at intraspecific level, which has allowed the selection of tolerant and productive genotypes for acidic soil[2].

Selection and breeding of Al-resistant genotypes are important for increasing seed yield in acidic soil. However, reliable ranking of tolerance in the field screening is difficult because of the temporal and spatial variation in acidic soil. Screening at field level is expensive and time-consuming when a large number of genotypes is under evaluation[3]. Therefore, a rapid and effective screening system is needed to discriminate tolerant and sensitive genotypes. The available screening methods for assessing Al tolerance in crops are based on the inhibition of root elongation in hydroponic culture and visual detection of Al tolerance levels by staining of seedlings root with hematoxylin[4]. However, reports on the use of hematoxylin staining and solution culture method in the screening of Al tolerance in pea are not available. In view of this, efforts were made to determine the effectiveness of the hematoxylin staining method for screening Al tolerance in pea at seedling stage.

Seeds of pea were soaked for one day and germinated in tap water. After 5 days, the seedlings were transferred and grown in nutrient solution having 0, 10, 20, 30 and 40 ppm Al (supplied as $AlCl_3 \cdot 6H_2O$). The pH of the nutrient solution was maintained at 4.5 for all treatments using 1 M HCl. The pH of the Al-treated nutrient solution was measured each day. Four plants per genotype were selected for uniformity in each of the triplicate trays for each Al concentration. After 24 days of growth, the roots were harvested separately and were given 20 s rinse in distilled water to remove surface contamination, followed by blotting to eliminate the entrained moisture. The effect of Al was expressed relative to the control (100%). Relative root length (RRL) was calculated using the formula: RRL (Root length with Al)/(Root length without Al) × 100.

The staining protocol was assayed following Polle *et al.*[4] with partial modifications for visual detection of Al in the roots. Seeds were presoaked in distilled water for 12 h and transferred to a filter paper to germinate in the growth chamber until the cotyledonary leaves emerged. Then the seedlings were transferred to plastic containers in nutrient solution (4.0 mM $CaCl_2$, 6.5 mM $KNO_3$, 2.5 mM $MgCl_2$, 0.1 mM $(NH_4)_2SO_4$, 0.4 mM $NH_4NO_3$) that was adjusted to pH 4.5 with 1 M HCl solution. Seedlings were kept in the above nutrient solution for 2 days under continuous light and aeration. The seedlings were then grown for 24 h on the fresh nutrient solution with 0–40 ppm Al concentration. The seedlings were placed in aerated distilled water for 60 min to remove Al on the root surface. The stain solution consisted of 2 g/l hematoxylin and 0.2 g/l $KIO_3$ which was prepared in distilled water. Trays containing the seedlings were immersed in hematoxylin staining for 30 min, after which the seedlings were transplanted in flowing distilled water for 30 min, three times. Each seedling was visually scored on the pattern of staining of the primary root tips. Seedlings were tested in completely randomized design with three replications for each Al level. Six seedlings per genotype per replication were visually scored: 0 = no staining, 1 = partial staining, 2 = moderate staining and 3 = deep staining. The 'no staining' and 'partial staining' seedlings were classified as tolerant, the 'moderate staining', seedlings as moderately tolerant, and those deeply stained as sensitive. Data were analysed using Minitab software.

Significant differences among Al levels and genotypes and their interaction were observed for RRL and root staining (Table 1), indicating differential response of genotypes to increasing Al concentration in RRL and root staining.

Symptoms of Al injury appeared first on the roots. Al-affected primary roots were short and stubby, and lateral roots become peg-like and the whole root system failed to elongate (Figure 1). The main cause for root length inhibition was suggested to be direct inhibition of cell division in the apical meristem. Inhibition of root elongation has been widely recog-


**Figure 1.** Effect of no aluminium (***a***) and 10 ppm Al (***b***) on the root system of pea seedling.


**Figure 2.** Staining of root tip of susceptible (***b***) and tolerant (***a***) genotypes at 30 ppm Al concentration by hematoxylin staining.

**Table 1.** Analysis of variance for relative root length (RRL; %) and score of root staining

| Source of variation | Degree of freedom | Mean square | |
|---|---|---|---|
| | | RRL | Score of root staining |
| Aluminium (Al) levels | 3 | 7249.8* | 15.33* |
| Genotype | 20 | 684.1* | 3.53* |
| Genotype × Al levels interaction | 60 | 73.6* | 0.15* |
| Error | 85 | 17.3 | 0.052 |

*Significant at 5% of probability.

nized as a trait for the screening of Al stress[5].

Considerable variation was found among the genotypes. RRL of all genotypes was severely reduced by Al toxicity. There was a significant negative correlation ($r = –0.681$*) between RRL and Al concentration. Tang and Keltjens[6] reported that Al toxicity was expressed by direct damage of the roots with a concomitant reduction in specific root length. Across all concentration genotype, PC-55-11-1-2 showed maximum RRL and thus was designated as the most tolerant, while genotype PC-493-5 exhibited lowest RRL and thus was the most tolerant. Earlier workers have also reported that root length is the more appropriate parameter for the Al tolerance ranking than root dry weight[7].

The correlation between RRL and hematoxylin staining for all genotypes showed a negative trend, which may be due to high Al accumulation in the sensitive seedlings. These results are in agreement with that of Cancaado[8], who reported a negative correlation between hematoxylin and RRL. At 30 ppm Al concentration, PC-55-11-1-2 was partially stained, whereas Arkel was deeply stained (Figure 2). Rincon and Gonzales[9] suggested that reduced staining of tolerant cultivars could be due to the presence of chelators.

The present findings suggest that 24 h exposure of Al is sufficient for discriminating Al-tolerant and sensitive genotypes using staining as a parameter. Giaveno and Miranda Filho[10] reported that 24 h of Al shock in roots was enough to result in staining when the roots were subsequently treated with hematoxylin.

Further, the results of RRL and root staining were compared, and drastic reduction in RRL was observed when complete staining of the root tips was attained. These results of relative order of Al tolerance based on the hematoxylin staining method are almost in agreement with Al tolerance order based on RRL in solution culture method, confirming that the hematoxylin staining method can be used for rapid screening because of its consistent performance.

As hematoxylin staining is a reliable, rapid and cost-effective screening technique, it can be used for the screening of a large number of pea genotypes at seedling stage for their Al tolerance. However, further investigations are needed to test the importance of seedling stage screening at adult plant stage with respect to growth and yield of pea genotypes.


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