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Five simple and rapid methods for evaluation of sorghum and pearl millet transgenics resistant to herbicide phosphinothricin (used as selectable marker) were studied. For rapid *in vitro* selection, three assays (establishment of sensitivity curves for embryogenic calli, determination of lethal doses for seed germination, and a rapid screening of cut young leaves based on the colour change of the medium) were established. For rapid screening of transgenic progeny, effects of *in vivo* Basta leaf spray and dip tests were studied at three different morphological stages. For all the above assays, LD50 and ID90 values were higher for pearl millet than sorghum. However, in both the crops, genotype effect was not significant. The assays standardized in the study were found to be effective for rapid, economical and mass-scale identification and characterization of transgenic plants of sorghum and pearl millet.

Limitations of application of gene transfer techniques to crops include the introduction, selection and expression of foreign genes in transgenics, especially monocotyledons1. The key to establish a successful transformation strategy lies in the adoption of an effective and foolproof selection strategy. The most common selection agents are antibiotics and herbicides2. A commonly used selection strategy for dicotyledons utilizes *neomycin phosphotransferase* II (*npt II*) gene isolated from *E. coli* that confers kanamycin resistance2.

Although this system is effective in tobacco and carrot transformation experiments3, it has proven to be less effective for monocot transformation.4,5,6. Earlier attempts to genetically transform sorghum used *npt II* gene conferring resistance to the antibiotic kanamycin6.7. Selection was not satisfactory because of natural resistance to kanamycin shown by sorghum and pearl millet cell cultures5,6,7. Also, due to detoxification of antibiotic by transformed cells, high frequencies of non-transformed rice and sorghum plants were obtained following selection with antibiotics2,7,8. Therefore, attempts were made to look for alternate markers for selection.

To date, the most successful and the most popular selection marker is the *bar* gene, derived from *Streptomyces hygroscopicus*, which encodes the enzyme phosphinothricin acetyltransferase (PAT) conferring resistance to the herbicide phosphinothricin (PPT) or its analogues Basta (with its active ingredient glufosinate ammonia) or bialophos. This herbicide has been used to obtain transgenic plants in most of the cereals including sorghum10,11 and pearl millet10,11.

Concentration of selection agent applied needs to be chosen very carefully. A too low concentration bears the risk of escapes. On the other hand, application of high concentrations of selection agents results in transplants with multiple copies of the transgene19. This is even more important in case of sorghum and pearl millet, where there is no well-established *Agrobacterium*-mediated transformation protocol. This optimal concentration for selection, in turn depends on the species19, which has to be evaluated experimentally, while taking into consideration the effect of post-transformation tissue damage on selection process20.

Besides the concentration, selection strategy depends on the marker gene used, and the type of explant transformed. Therefore, experiments to determine a dose-response curve are necessary before hand to arrive at the optimal concentration for efficient selection21. Hence, an important benchmark for using such transgenic selection strategy lies in the establishment of fool-proof evaluation systems before hand.

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For this, we have taken advantage of efficiency of bar gene for selection to confer PPT and Basta resistance in sorghum and pearl millet transgenic plants regenerated using different explants. A variety of strategies to study the transgenic expression in mature plant parts at biochemical and molecular levels are reported. However, many of these tests are expensive, time-consuming or ineffective when required to do on a large scale. Keeping this in view, we undertook this study to establish five simple, rapid and economical methods, three in vitro and two in vivo, for effective selection and screening of transgenics.

Materials and Methods

Plant materials—Three Sorghum genotypes - BTx-623, 296B and M35-1 and three pearl millet genotypes - 843-B, ICMP-451 and 7042-DMR were used for the study.

Six T₀ transgenic plants of sorghum (two from each genotype) and six T₀ transgenic plants of pearl millet (two from each genotype) confirmed by molecular analysis (PCR and southern) were taken for the study. T₀ plants were allowed to grow, self-fertilize, and set seed in the greenhouse. T₁ screening was performed using the five assay systems taking 40 progeny from each of the six T₀ transgenic events.

For all the experiments three replications were taken and each was repeated twice. The methodology involved in establishment of different assays is given in detail and the results obtained were statistically analyzed using Genstat statistical software.

Establishment of sensitivity curve with Basta for embryogenic calli—Sensitivity curve describes the dose response of embryogenic calli to Basta. To ascertain concentration of selection agent used in the tissue culture media that allow the selective growth of transformed cells, lethal doses were determined for 40 days old calli. Callus was transferred onto LS medium with 2,4-D (2.0 mg l⁻¹) and kinetin (0.2 mg l⁻¹) containing different concentrations of Basta (0, 0.5, 1, 2, 4, 6 and 8 mg l⁻¹). Selection agents were filter-sterilized before adding to different media. For each concentration, there were 3 replications, and there were 10 explants per plate. Plates were incubated in dark at 26°C. Transformation experiments in sorghum and pearl millet were performed as reported earlier and selection was performed using the lethal concentrations determined for non-transformed tissues.

Determination of lethal dose of Basta for germination and growth of seedlings—For surface sterilization, seeds of sorghum and pearl millet were dipped in 100 ml distilled water with 2 drops of Tween-20 and was washed for 10 min. Following rinsing thrice with sterile distilled water, seeds were placed in 70% ethanol for 1 min and then they were collected in a sterile flask. Seeds were rinsed again with sterile water for 5 min. After decanting the water, seeds were placed in HgCl₂ (0.1%) for 7 min under continuous stirring. Then, they were thoroughly rinsed again with sterile water twice for 10 min. Seeds were placed in petri plates on sterile filter paper soaked with Basta solution, sealed with parafilm, and were incubated in light (16 hr per day, 4000 Lux) at 26°C and 45% RH.

Seven levels of Basta solution (100, 200, 300, 500, 700, 1000, 2000 mg l⁻¹) dissolved in sterile distilled H₂O were used along with control (sterile distilled H₂O). For each treatment, one plate containing 25 seeds and 3 replications (a total of 75 seedlings per treatment) was used. Approximately, 5 ml of Basta solution was added to each plate under aseptic conditions. Percentage of germination was recorded at 24 hr intervals, over a period of 7 days starting from third day of treatment.

T₁ seeds of sorghum and pearl millet were germinated on medium with 1000 and with 2000 mg l⁻¹ of Basta respectively. Control experiments in a preliminary study indicated a total inhibition of germination at these Basta concentrations. Germination percentage was calculated in each category over a period of 7 days starting from third day of treatment as done in the control experiment.

Chlorophenol red assay to study the effect of Basta on pH of the medium in vitro—For chlorophenol red assay, we modified the protocol of Kramer et al.³⁴. Young leaves of greenhouse grown sorghum and pearl millet plants at 5 leaf stage were used for the experiment. With the help of scissors, leaves were separated from the plant, surface-sterilized and cut into pieces of 3 cm size. Leaf pieces were then transferred to petri plates at the rate of two pieces per plate, containing MS medium, benzylaminopurine (4 mg l⁻¹; BAP) and naphthalene-acetic acid (0.5 mg l⁻¹; NAA), with different concentrations of Basta (0, 4, 8, 12, 16 and 32 mg l⁻¹).

The medium was then poured at the rate of 20 ml per plate, and for each treatment, there were three replications. For all the concentrations, initial pH was noted. After four days of incubation period, pH was measured again. Chlorophenol red (25 μl of 0.5%; Flow laboratories, Scotland) was added to plates, and the gradation in colour intensity was noted visually.
Similarly, leaf pieces from T1 transgenics of sorghum and pearl millet were incubated in petri plates containing the above MS medium (25 ml) with Basta (32 mg l⁻¹), the concentration where greater pH changes as well as high leaf scorching was observed with non-transformed tissues. On addition of chlorophenol red, tissues resistant to Basta would develop yellow color, while susceptible tissues show pink to red coloration.

**Determination of dosage response of seedlings to Basta spray**—To ascertain Basta levels that are lethal to the growth of young sorghum and pearl millet plants, 7, 10, 14 days-old greenhouse germinated plants were used. Different levels of Basta solution dissolved in distilled water were sprayed along with control. For sorghum, 0, 0.5, 10, 100, 200, 300 and 500 mg l⁻¹ of Basta were taken. For pearl millet, 0, 25, 50, 75, 100, 200 and 300 mg l⁻¹ of Basta were taken. For each treatment, one pot containing average 18 (15-20) seedlings, and 2 replications were used. Approximately, 25 ml of solution was sprayed using a sprayer, from top to bottom of each pot. Observations were recorded at 24 hr intervals over a period of 7 days from the start of the treatment.

**Basta leaf dip assay**—The objective was to ascertain Basta levels that are lethal to fully expanded leaves at 5 leaf stage of sorghum and pearl millet plants grown in greenhouse.

Different levels of Basta solution dissolved in distilled water along with control were used. For sorghum, 0, 0.001, 0.005, 0.01, 0.025, 0.25, 0.5, and 1.0% of Basta, and for pearl millet, 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 4.0% of Basta were taken. For each treatment, one pot with 5 seedlings in 2 replications were used. Solution was applied by dipping upper 7.5 cm of the third and fifth leaves into a beaker (25 ml) three-fourth filled with Basta solution. Observations were recorded at 24 hr intervals over a period of 7 days from the start of the experiment. Area of the applied leaf that got scorched was accordingly recorded on 0 to 9 scale (0, no scorching; 9, 100% scorching) against the treatment given.

**Results and Discussion**

In this study, the optimal concentration of Basta that can fully inhibit the growth of non-transformed cells or tissues was determined for each of the five systems. For all the assays, except the Basta sprays, LD₉₀ and LD₁₀₀ values were significantly higher in pearl millet than sorghum (Table 1). However, for both the crops, the effect of genotype was found to be insignificant.

**Establishment of sensitivity curve with Basta for embryogenic calli**—Basta affected the growth of calli significantly and the effect was more in sorghum than in pearl millet. In sorghum, 50% of the explants died (LD₅₀) on medium with 1 mg l⁻¹ of Basta, and 100% mortality (LD₁₀₀) occurred at 3 mg l⁻¹ of Basta. With pearl millet, LD₅₀ and LD₁₀₀ values were obtained at 2 and 8 mg l⁻¹ of Basta, respectively (Figs 1A, 2A). Transformed calli were normal and light yellow on Basta medium, whereas, the non-transformed ones showed necrosis and cell death (Fig. 3A). The regenerants from surviving calli on further molecular analysis by PCR and southern hybridization were found to carry transgenes in them (data not reported here)⁵,⁶.

**Lethal dose of Basta for germination and growth of seedlings**—Presence of Basta in MS basal medium inhibited seed germination. The level of Basta in the medium had significant effect on germination percentage. With increasing Basta concentration, germination percentage decreased. This effect reached a plateau after 4 days. There was total inhibition of germination when 1000 mg l⁻¹ or above concentration of Basta was added to the medium in sorghum (Figs 1B, 2B). LD₅₀ was at 2000 mg l⁻¹ in pearl millet. However, genotypic effect within the crop was found to be statistically insignificant.

Germination percentage varied when transgenic seed was germinated on medium with Basta. Sorghum

<p>| Table 1 — Basta lethal dose (LD, at 50 and 100% mortality) values in different assay systems for sorghum and pearl millet |</p>
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seeds (T₀) showed 45-50% germination (Fig. 3B), while it was 43-52% in pearl millet. Further, seeds germinated on medium without Basta showed 100% germination. As the germinated seed represents T₁ generation, a segregation ratio of 75% resistance: 25% susceptibility was expected. However, the percentage germination observed was less than the expected, indicating that this could be due to difference in the level of transgene expression. Thus, this assay can help in easy identification of plants in which transgene will be expressed.

**Chlorophenol red assay to study the effect of Basta on pH of the medium in vitro** — In both the crops, at the end of 4 days of incubation, leaf pieces in the control plates remained green, while the pieces in Basta plates showed different levels of scorching.

Fig. 1 — *In vitro* and *in vivo* herbicide screening methods for sorghum and pearl millet. Response of sorghum and pearl millet to herbicide *in vitro* and *in vivo*. (A) Determination of lethal dose of herbicide Basta from the kill curve. Ten to fifteen calli were transferred to LS media containing different concentrations of Basta; (B) Inhibition of seed germination on medium with different concentrations of Basta; (C) Chlorophenol red assay to study the effect of Basta on pH of the culture medium. Leaf pieces of 3 cm size were incubated for 4 days in petri dishes containing MS salts + BAP (4 mg l⁻¹) + NAA (0.5 mg l⁻¹), with different levels of Basta. Values given are the means of pH difference, after 4 days on incubation; (D) Effect of Basta spray on the viability of 5 leaf staged seedlings. Approximately, 25 ml of the solution was sprayed per pot, and there were 2 pots per treatment; and (E) Basta leaf dip assay. Basta solution was applied by dipping upper 7 cm inches of the leaf in Basta solution.
Fig. 2— *In vitro* and *in vivo* assay systems for evaluation of herbicide phosphinotricin control plants. (A)-Embryogenic calli of pearl millet genotype 843-B on culture medium with Basta. Calli were cultured on medium with 6 levels of Basta along with control; (B)-Determination of effect of Basta on seed germination in sorghum genotype BTx-623. Inhibition of seed germination on a medium with different concentrations of the herbicide Basta. Arrows point out at seedlings on control plate (100% germination) and 1000 mg l⁻¹ Basta (total inhibition); (C)-Leaf pieces of pearl millet genotype ICMP- 451 on MS solution with different levels of Basta in mg l⁻¹ (indicated by numbers in the figure) showing the senescing leaves. At the end of incubation period, 25 μl of chlorophenol was added to the plate. Left arrow indicates Control (yellow in colour) and right 32 mg l⁻¹ of Basta plates (pinkish-red in colour); (D)-Effect of Basta spray (mg l⁻¹) on the viability of the seedlings in sorghum genotype 296B. Results of 2 leaf stage are shown in the front row and with 3-leaf stage in back row; and (E)-Effect of Basta on leaf viability when applied at 5 leaf stage of development in sorghum genotype M35-1. Basta when applied on the leaf reduced its viability. Area of leaf scorched increased with increase in the concentration of Basta, and concentrations of 0.5% or more showed scorching of the entire leaf.

In the control plate, pH decreased, while the plates with Basta showed a gradual increase, which was directly proportional to Basta concentration used (Fig.1C). Presence of Basta in the culture medium led to an increase in pH of the medium (alkalization) resulting from accumulation of ammonium ions to over 100-150-fold higher than in control plants. This increase towards alkalization is also made evident, by adding chlorophenol red, whose colour change is pH-dependent. Culture plates showed a gradation of colour from yellow in control plates to pink, red and deep red with increasing concentrations of Basta in the medium (Fig. 2C). Also, this assay can be performed at all the stages of genetic transformation, making the monitoring process simple and effective.

When leaf pieces from T₁ progeny of sorghum and millet transgenics were tested on medium with Basta (32 mg l⁻¹), browning was seen in some leaf pieces, while some other remained largely green over the same period. Further, when chlorophenol red was added to the plates, culture plates with green leaf pieces developed yellow color, while the plates with scorched leaf tips showed red to deep red coloration (Fig. 3C). Molecular analysis of these plants confirmed that leaf pieces that turned brown did not contain the *bar* gene, while the plants that remained green carried the *bar* gene (data not reported here). Thus, this assay is simple and easy to handle for mass scale screening of transgenics.

**Determination of dosage response of seedlings to Basta spray**—Spraying of Basta affected the viability of the seedlings. Viability of plants decreased with increasing concentration of Basta spray. In both the crops, LD₉₀ was observed at 50 mg l⁻¹ of Basta in 7 days-old seedlings (at 2 leaf stage of development). Complete inhibition of germination (LD₁₀₀) was observed when 100 mg l⁻¹ and above concentrations of Basta were used (Figs 1D, 2D). Resistance levels increased with increase in the age of the plants, and when 10 days-old plants (third leaf emerging) were
sprayed, Basta of 75 mg l⁻¹ was required to show LD₅₀. In case of two weeks old plant (fully expanded 3rd leaf), LD₅₀ was observed when 75 mg l⁻¹ of Basta was sprayed, and LD₁₀₀ when 100 mg l⁻¹ of Basta was sprayed both in sorghum (Fig. 2D) and pearl millet. However, the effect of genotype was not statistically significant.

Different levels of resistance to Basta were observed when regenerated transgenics were sprayed with lethal dose of Basta obtained through preliminary assays (Fig. 3D, E). T₁ sorghum transgenics showed a segregation ratio of 3:1 resistance: susceptibility. Similar results were obtained with T₁ progeny of pearl millet.²⁶

**Basta leaf dip assay**—Effect of Basta on the leaves was visible in the form of leaf scorching. This scorching increased with increase in the concentration of Basta applied (Figs 1E, 2E). Visual observations showed that more than 50% of the third leaf area scorched when 0.05% of Basta was applied in sorghum. However, in case of pearl millet 0.25% of Basta was required to show the same effect. At fifth leaf, a concentration of 0.1% produced the same extent of scorching in sorghum (0.5% in pearl millet). However, at higher concentrations (0.25%-0.5% in sorghum, and 2-4% in pearl millet), 100% scorching was observed in both the leaves. Even the non-treated adjoining (fourth and sixth leaves) leaves and plants died after 7 days of application (Fig. 1E).

A clear demarcation between susceptible and resistant plants was observed when Basta solution of 0.5% was applied to fifth leaf of sorghum T₁ plants (Fig. 3E). More than 90% of the leaf scorched in susceptible plant whereas the applied leaves remained green in resistant plant (Fig. 3E). The T₁ progeny tested segregated in the expected ratio (3:1) of resistant: susceptibility. Moreover, as compared to leaf paint assays, leaf dip assay ensures, more uniform application of

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**Fig. 3**—*In vitro* and *in vivo* assay systems for evaluation of herbicide phosphinothricin resistant transgenic plants (A)—PATGUS bombarded calli of pearl millet genotype 843-B on selection medium. 2 mg l⁻¹ of Basta was added to the growth medium. Only the transformed sectors were surviving (indicated by arrows), while non-transformed ones are dying; (B)—Sorghum T₁ transgenic seed (genotype BtX - 623) on Basta medium. Only the resistant seedlings are germinating; (C)—Leaf pieces of pearl millet T₁ progeny on MS solution with 32 mg l⁻¹ Basta showing resistant (plates 1 - 3) and susceptible transgenics (plates 4 - 6); (D)—Effect of Basta spray (75 mg l⁻¹) on T₁ generation of pearl millet transgenics of genotype 843-B at 2 leaf stage showing susceptible controls (left arrow) and segregating progeny (right arrow); and (E)—From left to right, control plant leaf treated with distilled water (left arrow), two resistant, and one susceptible T₁ plant (right arrow) of sorghum (genotype 296B) to which 0.5% Basta was applied.
herbicide, thereby reducing the chances of experimental errors.

Further, the results of these in vitro and in vivo assays showed high correlation with the PCR and Southern analysis of transgenics. We have already shown the practical applicability of these assays for evaluation of sorghum transgenics. As stable transformation occurs at very low frequency, an efficient selection strategy will help in recovery of maximum number of transformants. Thus, these assays will enhance the overall genetic transformation efficiency, and allow rapid, economic and mass scale screening of sorghum and pearl millet transgenics.

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