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*Standard Review*

# Millet improvement through regeneration and transformation

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**Millets, comprising the small-seeded group of the Poaceae family, represent one of the major food- and feed-crops in the semi-arid tropical regions of Africa and Asia. Compared to major crops of the world, these indigenous crops possess a number of beneficial characteristics including tolerance to extreme climatic and soil conditions; hence, adapts to poor soil fertility and moisture deficient areas. Moreover, millets are also nutritionally rich especially in vitamins and minerals, and most of them are gluten-free. Despite all these benefits, millets are encountered with several production constraints. The major bottleneck affecting millets are their extremely low yield since they are mostly cultivated in marginal areas with poor moisture and fertility conditions. Inherent characteristics, such as susceptibility to lodging, also significantly affect the productivity of millets. Millets are also commonly known as orphan- or neglected-crops due to too little attention given to them by the world scientific community. Genetic improvement in millets could be achieved not only by conventional approaches but also through modern techniques such as genetic modification or transgenics. The main benefits of regeneration and transformation in millet improvement are: i) the multiplication of identical copies of plants that are free of diseases and pests, and ii) the regeneration of the whole plant from transformed tissues with desirable traits. Success in plant transformation is largely dependent on the efficiency of regeneration. Establishing optimum regeneration method for each plant species and ecotype is therefore, a pre-requisite before embarking on plant transformation. In this review, we present various studies made to identify optimum regeneration and transformation methods for major millets. The prospects of applying advanced regeneration and transformation techniques to these vital but under-studied crops of the developing world are also discussed.**

**Key words:** Millets, under-researched crops, orphan crops, *in vitro* regeneration, transformation.

## INTRODUCTION

Millets represent the small-seeded group of the Poaceae family. The similarities of millets are that they are grown under extreme environmental conditions and therefore, especially suited to areas with inadequate moisture or short-growing cycle and poor soil fertility (Baker, 2003). Although millets are many in number, the most widely-cultivated ones are pearl millet [*Pennisetum glaucum* (L.) R. Br.], finger millet [*Eleusine coracana* (L.) Gaertn], tef [*Eragrostis tef* (Zucc.) Trotter], fonio or acha [*Digitaria exilis* (Kippist) Stapf and *D. iburua* Stapf], foxtail millet [*Setaria italica* (L.) P. Beauvois], proso millet [*Panicum miliaceum* (L.)], barnyard millet [*Echinochloa crusgalli* (L.)P. Beauvois] and kodo millet [*Paspalum scrobiculatum* (L.)].

Millets play key role in the maintenance of food security in the developing world since they are the major food and feed sources. Together with sorghum, millets account for about half of the total cereal production in Africa (Belton and Taylor, 2004). However, the average yield for millets is only 0.8 ton $ha^{-1}$ as compared to 3.5 ton $ha^{-1}$ for other cereals (FAOSTAT: http://faostat.fao.org/ accessed 21.12.2011). Millets are rich sources of human and livestock nutrition in developing countries (NAS, 1996).

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They contain high amount of vitamin, calcium, iron, potassium, magnesium, and zinc (Leder, 2004). In addition to being nutritious, millets are also considered as healthy food. The grains of most millets do not contain gluten (Leder, 2004), a substance that causes coeliac disease or other forms of allergies. Six millet species (namely, kodo-, finger-, proso-, foxtail-, little- and pearl-millet) were recently shown to have an anti-proliferative property and might have a potential in the prevention of cancer initiation (Chandrasekara and Shahidi, 2011), due to the presence and amount of phenolic extracts (Rao et al., 2011). Similar to maize and sorghum, millets follow the C4 photosynthesis system (Brutnell et al., 2010; Warner and Edwards, 1988); hence they prevent photorespiration and as a consequence efficiently utilize scarce moisture in the semi-arid regions.

To meet the strong increase in cereal demand worldwide, new approaches and technologies for generating new varieties are necessary. One of these methods is the creation of transgenic plants with desirable traits. Although millets are economically important, especially in the developing world, little genetic improvement has been done so far specifically using wide- or cross- hybridization among closely related species. The incompatibilities due to interspecific hybridization are alleviated by directly transferring the desirable traits to millets using optimum or efficient transformation method. Hence, crossing barriers could be overcome, and genes from unrelated sources would be introduced asexually into crop plants. Monocots in general and cereals in specific were initially difficult to genetically engineer, mainly due to their recalcitrance to *in vitro* regeneration and their resistance to *Agrobacterium*-mediated infection. However, efficient transformation protocols have been later established for the major cereals including rice and maize. Gene transfer to millets would be facilitated once efficient or optimum regeneration and transformation techniques are established.

The optimization of regeneration method is, therefore, necessary for different millet types in order to increase the efficiency of transformation. In this review, we present various regeneration and transformation techniques studied for major millets. We also discuss the prospects of applying advanced techniques developed for major cereals to millets, vital but understudied crops of the developing world.

## REGENERATION STUDIES IN MILLETS

The first regeneration studies in millets were performed in the 1970s for proso-, finger-, pearl- and kodo- millets (Rangan, 1973, 1976). Subsequent investigations were also made for other millet species. In the following sections, key parameters affecting millet regenerations are reviewed (Table 1). These important factors include explants, plant growth regulators (PGRs) and media. Although environmental factors such as temperature, pH and light also affect the regeneration processes, they are not discussed here.

### Regeneration processes

Plant regeneration is achieved by the process of either somatic embryogenesis or organogenesis. Somatic embryogenesis relies on plant regeneration through a process similar to zygotic embryo germination. Somatic embryos are developed either directly or indirectly through an intermediate step of callus formation. Direct embryogenesis occurs in plants rarely, compared to the indirect somatic embryogenesis.

The organogenesis process relies on the production of organs either directly from an explant or from a callus culture. It is a rare event in millets; to date, only finger millet and pearl millet were regenerated through organogenesis (George and Eapen, 1990; Jha et al., 2009).

### Explants for regeneration

Explants refer to sterile pieces of the plant from which regeneration is initiated. The suitability of explants for regeneration depends on the type of the genotype and the culture media used. The maximum callus inductions obtained from different explants of pearl- and finger-millet are shown in Figure 1. Identifying the best explant is critical for increasing the competence of plant regeneration.

Roots were used as an explant in tef and finger millet. While the callus induction was more than 90% in finger millet (Mohanty et al., 1985) (Figure 1B), in tef, a maximum of 25% callus formation was obtained (Bekele et al., 1995). The difficulty of using root as an explant was also reported for major cereal crops such as wheat and barley (Bhojwani and Hayward, 1977; Chin and Scott, 1977). Another easily available explant is the shoot apical meristem (SAM) which contains the zone of actively dividing cells. The suitability of SAM as an explant was demonstrated in finger millet and pearl millet (Eapen and George, 1990; Lambe et al., 1999). Mesocotyl, the plant part between the cotyledon and the coleoptile, was also used as an explant for finger- (Rangan, 1976; Mohanty et al., 1985; Eapen and George, 1990), proso- (Rangan, 1973; Heyser and Nabors, 1982), kodo- (Rangan, 1973), and pearl-millet (Rangan, 1976).

Mature seeds and embryos were also studied in most millet types although mature embryos generated lower percentage of somatic embryos than immature embryos in kodo- and pearl- millet (Vikrant and Rashid, 2002b; Goldman et al., 2003; Campos et al., 2009). Immature inflorescences were also evaluated for their regenerative response especially in pearl millet where they gave the

**Table 1.** Summary of *in vitro* regeneration studies for important millets regarding explants, regeneration processes and growth regulators.

| Millet type (species) | Explant | Processes | Growth regulators | Reference |
|---|---|---|---|---|
| Pearl millet (*Pennisetum glaucum*) | Mesocotyl | Somatic embryogenesis<br>Plant regeneration | 2.4-D<br>IAA | Rangan (1976). |
| | Immature inflorescence; immature embryo; mature seed; leaf segment and shoot tip | Somatic embryogenesis | 2,4-D or pCPA alone or with KIN or BA | Vasil and Vasil (1981, 1982), Pius et al. (1993), Lambe et al. (1995, 1999, 2000), Mythili et al. (1997), Oldach et al. (2001), Girgi et al. (2002, 2006), Srivastav and Kothari (2002), Goldman et al. (2003), O'Kennedy et al. (2004, 2011a, 2011b), Satyavathi et al. (2006), Muthuramu et al. (2008) and Jha et al. (2009). |
| | | Plant regeneration | $GA_3$; BA or ABA alone; IAA with KIN or BA with IAA or KIN or TDZ or 2,4-D | |
| | | Root formation | NAA alone or IBAor IAA; KIN | |
| | | Organogenesis | BA | |
| | Mature embryo | Somatic embryogenesis | 2,4-D | Campos et al. (2009). |
| | Root, mesocotyl and leaf base | Somatic embryogenesis<br>Plant regeneration | 2,4-D<br>None or NAA | Rangan (1976) and Mohanty et al. (1985). |
| | Shoot tip, immature inflorescence and mesocotyl | Somatic embryogenesis<br>Plant regeneration<br>*Organogenesis* | 2,4-D or picloram; KIN or BA[2]<br>KIN with TDZ or IAA<br>2,4-D; zeatin[2] | Eapen and George (1990), George and Eapen (1990), Latha et al. (2005) and Ceasar and Ignacimuthu (2008). |
| Finger millet (*Eleucine coracana*) | Mature seed | Somatic embryogenesis | 2,4-D alone or with KIN | Sivadas et al. (1990), Poddar et al. (1997), Gupta et al. (2001), Kothari et al. (2004), Kothari-Chajer et al. (2008), Nethra et al. (2009) and Sharma et al. (2011). |
| | | Plant regeneration | $GA_3$, BA or NAA alone or KIN; IAA | |
| | Mature embryo and epicotyl | Somatic embryogenesis<br>Plant regeneration<br>Root formation | 2,4-D<br>BA or KIN<br>IBA; BA | Patil et al. (2009). |
| | Leaf and root explant and mature seed | Callus induction<br>Somatic embryogenesis | 2,4-D or 3,6-D or dicamba<br>2,4-D or 3,6-D or dicamba; ABA, BA; KIN | Bekele et al. (1995) and Mekbib et al. (1997). |
| Tef (*Eragrostis tef*) | Mature seed | Somatic embryogenesis<br>Embryo promotion<br>Plant regeneration | 2,4-D followed by TIBA<br>2,4-D; KIN followed by IAA; BA<br>$GA_3$ | Assefa et al. (1998). |
| | Immature spikelet and panicle segment | Gynogenic tissue induction | 2,4-D; BA | Gugsa et al. (2006) |

**Table 1.** Contd.

| | | | | |
|---|---|---|---|---|
| | Immature anther and embryo | Somatic embryogenesis<br>Plant regeneration | 2,4-D<br>BA alone or with NAA | Tadesse et al. (2009) and Gugsa and Kumlehn (2011). |
| Fonio (*Digitaria exilis*) | Stem segment | Somatic embryogenesis<br>Shoot development | 2,4-D<br>BA; $GA_3$[1] | Ntui et al. (2010). |
| Barnyard millet (*Echinochloa crusgalli*) | Mature seed | Somatic embryogenesis | 2,4-D[2] | Gupta et al. (2001). |
| Proso millet (*Panicum miliaceum*) | Immature and mature embryo, mature seed, immature inflorescence, mesocotyl, shoot tip and leaf and stem segment | Somatic embryogenesis<br>Plant regeneration | 2,4-D alone or with KIN[2]<br>2,4-D or NAA | Rangan (1973), Bajaj et al. (1981), Heyser and Nabors (1982), Rangan and Vasil (1983) and Heyser (1984). |
| Kodo millet (*Paspalum scrobiculatum*) | Immature inflorescence, immature and mature embryo; mature seed; young leaf base and mesocotyl | Somatic embryogenesis<br>Shoot regeneration<br>Root formation | Picloram or 2,4-D alone or with TDZ[1]; KIN<br>NAA alone or with BA<br>PAA | Rangan (1976), Nayak and Sen (1989, 1991), Vikrant and Rashid (2001, 2002a, 2002b, 2003), Kaur and Kothari (2004) and Kothari-Chajer et al. (2008). |
| | Shoot tip | Somatic embryogenesis<br>Plant regeneration<br>Root formation | 2,4-D alone or with KIN<br>TDZ alone[1] or BA; NAA<br>IBA | Arockiasamy et al. (2001) and Ceasar and Ignacimutu (2010). |
| Foxtail millet (*Setaria italica*) | Immature inflorescence; mature embryo; mature seed and shoot tip | Somatic embryogenesis<br>Plant regeneration | 2,4-D alone or with KIN or BA<br>NAA with BA or KIN or 2,4-D with KIN | Xu et al. (1984), Rao et al. (1988), Reddy and Vaidyanath (1990), Osuna-Avila et al. (1995), Liu et al. (2005), Qin et al. (2008) and Wang et al. (2011). |

[1] Transfer to medium without any growth regulator for root formation.
[2] Transfer to medium without any growth regulator for plant regeneration.

highest percentage of somatic embryos and shoot regeneration compared to shoot tips and seeds (Jha et al., 2009). Moreover, anthers were successfully used as an explant in tef (Tadesse et al., 2009). In general, immature embryos are the main source of an explant not only in the major cereal crops but also in millets. In pearl millet alone, about 50% of the studies on regeneration used explants from immature embryo. In our laboratory, we routinely use immature embryos as an explant in order to regenerate tef (Figure 2A). We found that about 45% of immature embryos induced somatic embryos and about 55% of these somatic embryos formed plantlets (Figure 2B).

## Plant genotypes

The types of genotypes or crop cultivars also determine the efficiency of regeneration. The investigation made on eight tef ecotypes indicated that although variations in calli weight were negligible, differences in the percentage of

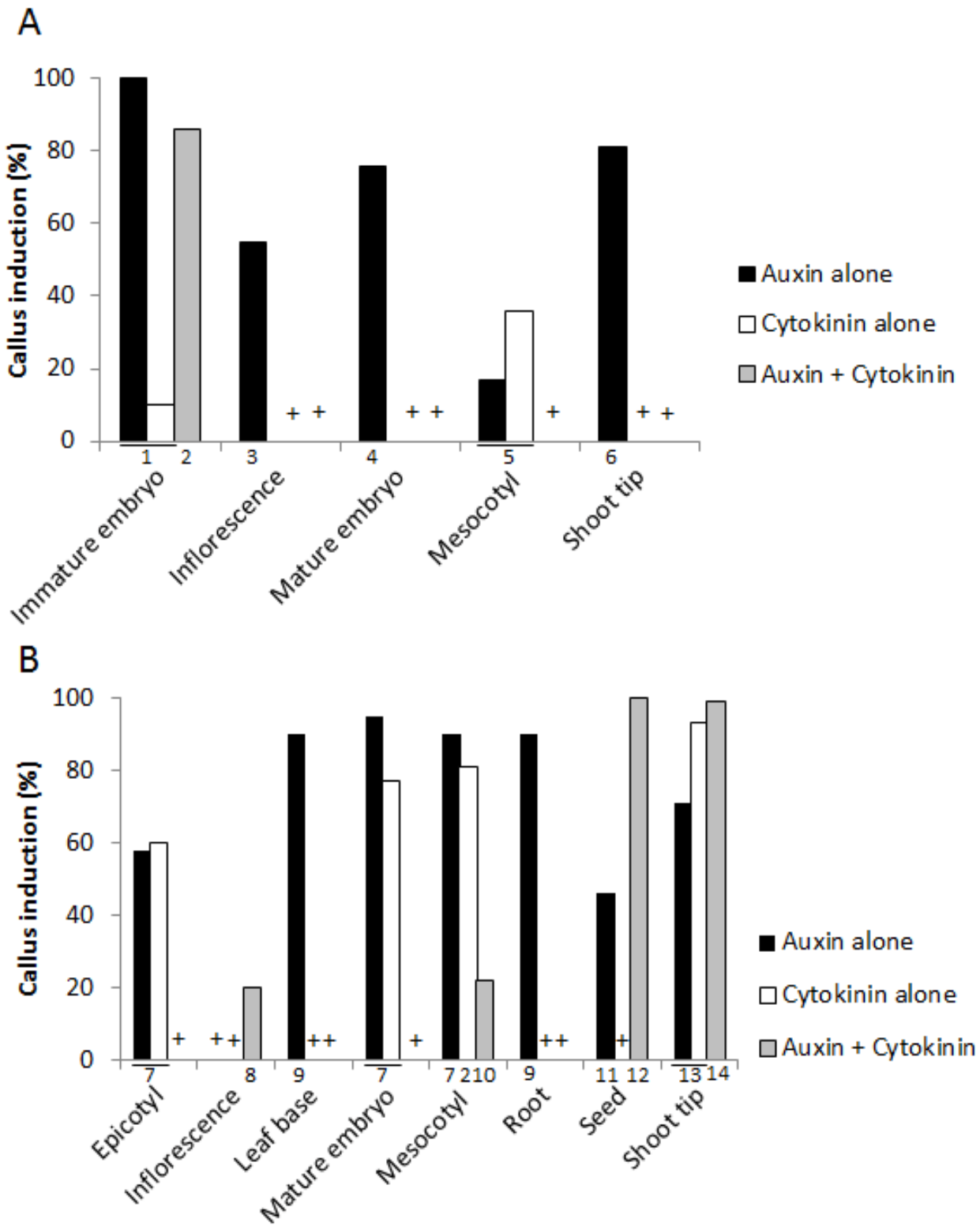


**Figure 1.** The maximum efficiency of callus formation in pearl millet (A) and finger millet (B). The callus induction for different explants depends on the type of hormone (auxin or cytokinin alone or by adding the two hormones together) applied. Data non-available or non-quantified were indicated as "+". The figure was made based on the results of the following authors: (1) Goldman et al. (2003), (2) Oldach et al. (2001), (3) O'Kennedy et al. (2004), (4) Campos et al. (2009), (5) Rangan (1976), (6) Lambe et al. (1999), (7) Patil et al. (2009), (8) George and Eapen (1990), (9) Mohanty et al. (1985), (10) Eapen and George (1990), (11) Gupta et al. (2001), (12) Kothari et al. (2004), (13) Ceasar and Ignacimuthu (2008) and (14) Latha et al. (2005). Numbers in the figure correspond to the references indicated.

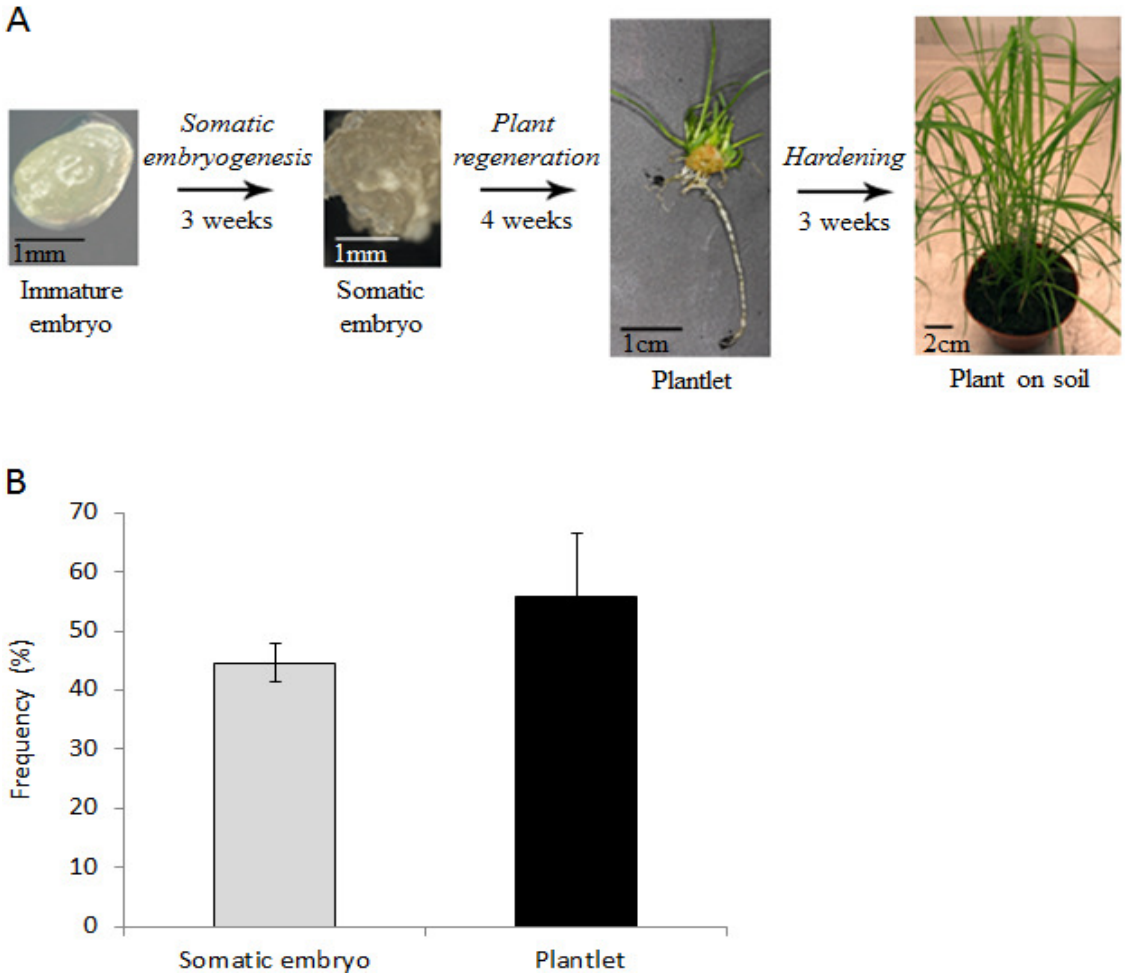


**Figure 2**. *In vitro* regeneration of tef. (A) Tef variety *Tsedey* (also known as DZ-Cr-37) was used for *in vitro* regeneration based on Gugsa and Kumlehn (2011). Immature embryos were placed on K99 medium (Deutsch et al., 2004) facing the scutellum side up and allowed to grow in the dark for 3 weeks in the presence of 90 g/l maltose, 1 g/l glutamine and 2 mg/l 2,4-D. Somatic embryos formed were transferred to K4NB medium (Kumlehn et al., 2006) in the light with 36 g/l maltose, 0.15 g/l glutamine and 0.22 mg/l BAP. After four weeks, plantlets were transferred to soil and grown first for three weeks in the long-day (16 h light: 8 h dark) followed by short-day (8 h light: 16 h dark) until harvesting the seeds. (B) Percentage (+/- standard error) of immature embryos transformed to somatic embryos and somatic embryos transformed to plantlets. One hundred fifty initial explants were used for the experiment.

regenerants were considerable among the ecotypes tested (Bekele et al., 1995). Despite huge expected variability among different ecotypes or cultivars, most regeneration experiments in millets use a single line or cultivar without testing its performance. Hence, obtaining a genotype with high regenerative capacity is a widespread problem in millet improvement.

## Plant growth regulators (PGRs)

PGRs are critical in determining the deve-lopmental pathway of the plant cells. Their roles in regeneration have been studied since the initial observations by Skoog and Miller (1957) half a century ago. Auxins and cytokinins are the most widely employed PGRs in plant regeneration. They are usually applied together in the medium as the ratio of auxin to cytokinin determines the type of organ or tissue to be regenerated. While high auxin to cytokinin ratio promotes root development, low ratio stimulates shoot development. The intermediate ratio, on the other hand, facilitates the formation of undifferentiated organ called callus. This paradigm was also confirmed in somatic embryogenesis of millets (Kaur and Kothari, 2004).

On the other hand, somatic embryogenesis was also obtained by the application of auxin or cytokinin alone (Figure 1). Earlier studies showed that for the long-term

maintenance of the callus, the concentration of auxin in the form of 2,4-D (2,4-Dicholorophenoxyacetic acid) and pCPA (*p*- chlorophenoxyacetic acid) need to decrease in finger millet and pearl millet, respectively (Sivadas et al., 1990; Kumar et al., 2001; Srivastav and Kothari, 2002) as prolonged exposure of cell cultures to high concentrations of auxin resulted in poor regeneration and caused chromosomal abnormalities (Deambrogio and Dale, 1980; Nabors et al., 1983). While in the majority of the somatic embryogenesis, 2,4-D was used as an auxin supplement, other types of auxin also showed good performance. Among these, picloram, a very potent growth regulator that induces somatic embryogenesis, was found to be superior to 2,4-D in kodo millet regeneration (Kaur and Kothari, 2004). A study in tef showed that the efficacy of auxin was dependent on the type of explant in which 2,4-D was best suited for leaf and root segments while 3,6-D for mature seeds (Bekele et al., 1995).

Shoot development was in the majority of cases formed once embryogenic calli were transferred to medium with a low auxin to cytokinin ratio (Girgi et al., 2002). However, many other reports indicated that successful regeneration were obtained by applying cytokinin alone in finger millet (Sankhla et al., 1992; Latha et al., 2005; Ceasar and Ignacimuthu, 2008; Yemets et al., 2003; Nethra et al., 2009), kodo millet (Ceasar and Ignacimuthu, 2010) and pearl millet (Mythili et al., 1997; Goldman et al., 2003; Satyavathi et al., 2006). Other workers also indicated that regeneration was promoted in diverse types of millets using either gibberellic acid alone (Sivadas et al., 1990; Nayak and Sen, 1991; Assefa et al., 1998; Kumar et al., 2001; Sharma et al., 2011) or together with cytokinin (Ntui et al., 2010). On the other hand, several other studies indicated that none of the known PGRs were necessary to regenerate shoots in finger millet (Eapen and George, 1990), proso millet (Heyser and Nabors, 1982), kodo millet (Vikrant and Rashid, 2001, 2002b) and pearl millet (Campos et al., 2009).

In general, in about half of regeneration studies on millets, PGRs were applied together in order to initiate shoots and roots simultaneously while in the remaining studies shoots were allowed to develop first followed by roots.

### Culture media

The composition of the culture medium is another important parameter that determines the efficacy of regeneration independent of the explant. The medium has to supply all essential nutrients necessary for the growth and development of the plant.

Most *in vitro* culture studies use Murashige and Skoog (or commonly known as MS) medium (Murashige and Skoog, 1962). However, the N6 medium (Chu et al., 1975) became popular in pearl millet since increased amount of embryogenic callus was obtained during long-term culturing (Lambe et al., 1999). In addition, compared to the MS medium, lower amount of auxin was required for the N6 medium (Vikrant and Rashid, 2001, 2002b).

Since the majority of plant cells are not photosynthetic, it is essential to add to the culture medium, a fixed carbon source. The type and concentration of carbon source also determine the competence of embryogenic calli to be formed. Carbon does not only serve as an energy source but also influences the osmolarity of the medium. Although sucrose is commonly applied in most tissue culture studies involving millets, maltose is preferentially used in pearl millet and tef (O'Kennedy et al., 2004; Tadesse et al., 2009; Gugsa and Kumlehn, 2011).

The concentration of micro-nutrients added to MS medium also affects the regeneration processes. The addition of higher concentration of cupric sulphate improved somatic embryogenesis, maintenance and regeneration in finger millet (Kothari et al., 2004). Another important component of the media is the ratio between nitrate and ammonia. Successful regeneration was reported in finger millet using high nitrate to ammonium ratio replacing PGRs (Poddar et al., 1997).

Organic compounds such as casein hydrolysate, glutamine and L-tryptophan were also proved to improve the initiation of embryogenic cultures in finger millet (Yemets et al., 2003). Although, the aforementioned report indicated the beneficial effects of amino acids on regeneration, another study in finger millet showed an adverse effect of certain amino acids on the initiation of shoots (Eapen and George, 1990).

Charcoal, which absorbs inhibitory compounds (Thomas, 2008), was also shown to increase the regeneration capacity in kodo millet and pearl millet (Vikrant et al., 2001; Lambe et al., 1999). Furthermore, ethylene inhibitors such as silver nitrate improved the regeneration process in pearl millet mainly by promoting the shoot formation (Pius et al., 1993; Oldach et al., 2001). Other ethylene inhibitors such as cefotaxime, carbenicillin and streptomycin similarly enhanced plant differentiation from somatic embryos in finger millet (Eapen and George, 1990). Cefotaxime and ASA (O-acetyl salicylic acid), another ethylene inhibitor, also enhanced regeneration efficiency in pearl millet (Pius et al., 1993).

## TRANSFORMATION STUDIES IN MILLETS

Genetic engineering or transformation refers to the delivery of DNA, encoding a desirable trait to the plant cell. In order to deliver pieces of DNA to the plant of choice, two methods, namely physical and biological, are used. The physical method includes particle or microprojectile bombardment and electroporation while the only successfully applied biological technique is

*Agrobacterium*-mediated transformation. Since both physical and biological methods facilitate the transfer of the traits of importance to the plants of interest, a number of crop improvement studies benefited from the technique. Traits commonly employed in the transformation are those which increase resistance against biotic and abiotic stresses or those which improve the quality of food.

In cereal crops, *in vitro* regeneration is an essential component of the transformation because optimum transformation could not be achieved without having a reliable regeneration protocol. Cereals were until recently difficult to genetically engineer, mainly due to their recalcitrance to regeneration and their resistance to *Agrobacterium* infection. In developing optimum transformation techniques for millets, the following points need to be considered: suitable explants, appropriate transformation method, and appropriate promoters and selectable markers (Repellin et al., 2001; Kothari et al., 2005).

Optimum transformation methods have been studied for some millets (Table 2). Several of these transformations targeted agronomically important traits including resistance to pathogens (Latha et al., 2005, 2006; Girgi et al., 2006; O'Kennedy et al., 2011a).

### Explants for transformation

Transformations of millets were largely dependent on embryogenic callus derived from seedlings, shoot tips, immature inflorescences and embryos, and mature seeds. However, initial explants such as leaf segments, pollen grains and immature embryos were also directly used in the transformation (Dong et al., 1999; Gupta et al., 2001; Girgi et al., 2002, 2006; Schreiber and Dresselhaus, 2003; O'Kennedy et al., 2004, 2011a, 2011b). The use of immature embryos instead of somatic embryos was found to be an ideal target for transformation of recalcitrant crop species especially cereals (Bartlett et al., 2008).

### Transformation methods

Irrespective of the type of explant, most millet transformations applied either the microprojectile bombardment or the *Agrobacterium*-mediated method of transformation. These methods require specific conditions to boost the efficiency of transformation. For instance, osmotic treatment of the explant with sucrose was found to improve the gene delivery system in pearl millet transformed by microprojectile bombardment (Goldman et al., 2003). The *Agrobacterium*-mediated transformation is dependent on the choice of appropriate strain. The most widely used *Agrobacterium* strain for millet transformations are *LBA4404, EHA101* and derivatives of *EHA101* (namely *EHA105, AGL0* and *AGL1*). In foxtail millet transformation, *LBA4404* performed significantly better than *EHA105* (Wang et al., 2011).

Although, *Agrobacterium*-mediated trans-formation is widely applied in cereals (Schrawat and Lörz, 2006), microprojectile bombardment is still the dominant method of transformation in millets despite its drawbacks, which includes multiple integration of the transgene into the target genome.

### Promoters and selectable markers

The type of promoter used for driving the gene of interest has significant impact on the efficiency of transformation. While the CaMV 35S (commonly known as 35S) promoter works perfectly in dicots, it has a low activity in monocots (McElroy and Brettell, 1994). Among five promoters tested for finger millet transformation, the Actin 1 promoter isolated from rice and the ubiquitin 1 promoter from maize gave the highest transformation efficiency (Gupta et al., 2001). In barnyard millet, however, only ubiquitin 1 was effective (Gupta et al., 2001).

Plant transformation also requires the proper choice of the selectable marker(s). Commonly used selectable markers are antibiotic- and herbicide- resistance. These selectable markers also enabled millet researchers to identify the right transformants. The applicability of hygromycin and kanamycin markers were also tested on protoplast cultures derived from pearl millet (Hauptmann et al., 1988). Another selectable marker recently developed from the *phosphomannose isomerase* (*manA*) gene showed promising performance in pearl millet (O'Kennedy et al., 2004). Transgenic *manA* expressing cells acquired the ability to convert mannose 6-phosphate to fructose 6-phosphate while the non-transgenic cells lose the ability to convert this product, and eventually die due to excessive accumulation of mannose 6-phosphate which is toxic if present in high amount. A recently developed technique in which a modified alpha-tubulin gene was used as a selectable marker in the form of herbicide resistance, gave good performance in finger millet transformation (Yemets et al., 2008).

## NEED FOR EFFICIENT REGENERATION AND TRANSFORMATION OF MILLETS

Regeneration has been studied since long time in diverse millet species. Rangan was a pioneer to investigate and successfully regenerate viable plants at least from three economically important millets, namely proso-, finger- and kodo- millets (Rangan, 1973, 1976). Later, optimum regeneration methods were also studied for other millets. Establishing efficient regeneration system requires optimization of various factors including the right type of explant and the proper composition of the medium. Compared to major cereals such as wheat and rice, little advancement was made in millet regeneration.

**Table 2.** Summary of transformation studies for economically important millets regarding explants, and method and purpose of transformation.

| Millet type (species) | Initial explant | Transformed explant | Method[1] | Promoter[2] | Purpose[3] | Reference |
|---|---|---|---|---|---|---|
| Pearl millet (*Pennisetum glaucum*) | Immature embryo; pollen grain and shoot tip | Immature embryos; embryogenic cell suspension; embryogenic callus; pollen grain and shoot-tip clump | MB | Enhanced CaMV 35S, ZmAdh1, ZmUbi, CaMV 35S, OsAct, ZmMADS2 | T | Taylor and Vasil (1991), Taylor et al. (1993), Dong et al. (1999), Devi and Stricklen (2002) and Schreiber and Dresselhaus (2003) |
| | Shoot tip; immature embryo; mature embryo and immature inflorescence | Embryogenic cell suspension, embryogenic callus, mature embryo | MB | CaMV 35S, ZmAdh1, Emu, ZmUbi, OsAct, double CaMV 35S, pin2 | S | Lambe et al. (1995, 2000), Girgi et al. (2002, 2006), Goldman et al. (2003), O'Kennedy et al. (2004, 2011a, 2011b) and Latha et al. (2006) |
| Finger millet (*Eleucine coracana*) | Mature seed and shoot tip | Embryogenic callus | MB | ZmUbi, CaMV 35S, OsAct, RbcS, ppcA-L-Ft | S | Gupta et al. (2001), Latha et al. (2005) and Yemets et al. (2008) |
| | Mature seed | Green nodular callus | A (EHA105) | CaMV 35S | S | Sharma et al. (2011) |
| Barnyard millet (*Echinochloa crusgalli*) | Mature seed and leaf segment | Embryogenic callus and leaf segment | MB | ZmUbi, CaMV 35S, OsAct, RbcS, ppcA-L-Ft | S | Gupta et al. (2001) |
| Guinea grass (*Panicum maximum*) | Cell line | Protoplasts | E | CaMV 35S | T | Hauptmann et al. (1987, 1988) |
| | Immature embryo | Embryogenic cell suspension and embryogenic callus | MB | ZmAdh1, ZmUbi | T | Taylor et al. (1993) |
| Foxtail millet (*Setaria italica*) | Immature inflorescence | Embryogenic callus | A (LBA4404; EHA105) | Zm13, PF128 | S | Liu et al. (2005), Qin et al. (2008) and Wang et al. (2011) |

[1] A: *Agrobacterium* transformation; E: Electroporation; MB: Microprojectile bombardment
[2] CaMV 35S: Cauliflower Mosaic Virus 35S; OsAct: rice actin ; ZmAdh1: maize alcohol dehydrogenase 1; Emu: engineered based on truncated Adh1; pin2: potato proteinase inhibitor IIk (wound inducible); ppcA-L-Ft: Flaveria trinervia phosphenolpyruvate carboxylase ; RbcS: rice small subunit of ribulose 1,5-biphosphate carboxylase; ZmMADS2: maize MADS-box gene 2 (pollen specific); ZmUbi: maize ubiquitin.
[3] S: Stable transformation of plants; T: Transient expression.

This was mainly because millets are crops of developing world that are limited by resources; hence investment towards improving these crops using tissue culture or regeneration, and transformation techniques is little advanced. As a result, these vital crops of resource-poor people in developing world did not benefit from agricultural revolutions such as Green Revolution that boosted the productivity of major crops. Once optimum transformation methods are established for millets, valuable agronomic and nutritional traits could be routinely transferred. Traits that

needed to be incur-porated to millets include resistance to biotic (for example, pathogens) and abiotic stresses (for example, drought), biofor- tification of useful nutritional elements, and altered architecture of the plant (for example, semi-dwarfism).

**Lessons from major cereals or model millets**

Advances made for major cereals in the area of regeneration and transformation could be applied to millets either directly or after some optimization. Some tissue culture techniques developed for model millets such as large crabgrass millet (*Digitaria sanguinalis*) could also be transferred to less researched millets.

Regeneration method that uses immature embryo as an explant is dominantly applied in monocots; hence it has also prospects in millets. The main problem associated to using immature embryos or inflorescences is the need for continuous growth of donor plants. Therefore, efforts should be made to investigate for alternative explants regarding accessibility, quantity, and cost. Leaves are the most common source of explant especially in callus initiation and subsequent plant regeneration in dicot plants. Unlike dicots, the vegetative parts of monocots do not readily proliferate; hence no successful regeneration was reported for cereals when leaves were used as an explant (Saalbach and Koblitz, 1978). However, due to its continuous growth similar to the meristematic region, the basal part of the leaf was successfully used in sorghum and wheat regeneration (Wernicke and Brettell, 1980; Wernicke and Milkovits, 1984). Explants such as the transverse thin cell layers (tCLPs) did not only boost the regeneration capacity in recalcitrant genotypes of rice, sorghum and maize (Nhut et al., 2003) but also in the non-food millet called large crabgrass millet (Le et al., 1997, 1998).

Moreover, the regenerative competence of the genotype should be considered while choosing the appropriate explant as different genotypes of same species show huge variability. Hence, appropriate regeneration techniques need to be established at least for economically important millets. A large scale screening methodology has to be developed in order to determine the regeneration capacity for diverse genotypes of millets as it was investigated for rice (Dabul et al., 2009). Another important point in regeneration is the prolongation of the viability period of the explant. TDZ (thidiazuron), a cotton defoliant with cytokinin-like activity, was found to increase the viability by shortening the somatic embryogenesis phase (Mok et al., 1982). TDZ has been used for the enhancement of morphogenic competence in Poaceae since mid-1990 (Wenzhong et al., 1994). The beneficial effect of TDZ on shoot bud development was observed in millets such as kodo millet, finger millet and switchgrass (Gupta and Conger, 1998; Vikrant and Rashid, 2002; Ceasar and Ignacimuthu, 2008).

Another important parameter affecting the efficiency of *in vitro* regeneration is the composition of the culture medium. Diverse types of media were shown to improve regeneration in major crops (Wang et al., 1993; Kumlehn et al., 2006) and millets (Heyser, 1984; Nayak and Sen, 1991; Latha et al., 2005; Gugsa and Kumlehn, 2011). The type and concentration of carbon source affect the efficacy of regeneration. For example, an increased osmolarity due to sucrose, sorbitol, mannitol and maltose showed to improve embryo formation and maintenance in maize (Lu et al., 1983). The replacement of sucrose by maltose increased the efficiency of embryogenesis and regeneration in wheat and tef (Mendoza and Kaeppler, 2002; Gugsa and Kumlehn, 2011).

Optimum regeneration techniques targeting the rescuing of the progenies of crosses between economically important millets and their wild relatives need to be investigated in order to introduce important agronomic traits to the cultivated species. These introgressions between divergent species require a special regeneration procedure known as embryo rescue, a technique which allows the hybrids to become fertile. Although embryo rescue techniques are widely applied in crop plants (Sharma and Ohm, 1990; Price et al., 2005), they are not yet developed for millets.

Significant developments have also been made in transformation of major cereals (Repellin et al., 2001). However, optimum transformation methods are not yet established for most millet species. Although *Agrobacterium*-mediated transformation is becoming the main mode of transformation for major cereals (Komari and Kubo, 1999; Koichi et al., 2002) especially due to its simple integration in the plant genome, it is not widely practiced in millets. On the contrary, the microprojectile bombardment method is the dominant transformation technique in millets despite its pitfalls especially related to complex integration pattern of the transgene in the plant genome. Improvement in *Agrobacterium*-mediated transformation was achieved by applying acetosyringone in both the transformation and co-cultivation media. The addition of acetosyringone and cell extracts from dicot plants during the co-cultivation process increased the transformation efficiency of rice (Hiei et al., 1994) and recently also in millets (Liu et al., 2005; Sharma et al., 2011).

Another important point to be considered in monocot transformation is the selection of the right promoter. Ubiquitin and actin promoters are widely used in cereals transformation. Recently, two ubiquitin promoters, namely Ubi 1 and Ubi 2, which were isolated from switchgrass (*Panicum virgatum* L.), resulted in strong expression of reporter gene (Mann et al., 2011). In addition, except in few cases, transformation studies in millets did not address important agronomic problems or traits. Based on the available literature, the only two food-security important millets in which transformation was focused on transferring agronomically valuable traits were pearl millet and finger millet (Latha et al., 2005, 2006; Girgi et al.,

2006; O'Kennedy et al., 2011a).

## CONCLUSION

In general, millets play huge role in the livelihood of the population of developing world especially due to their enormous contribution to food security. However, since these crops are not sufficiently studied, for which the name orphan crops is given to these groups of crops, they remain largely unimproved. Both conventional and modern improvement techniques were not adequately implemented. The regenerative competence of the explant should be considered while choosing the appropriate explant as different genotypes of same species show enormous variability in regeneration. Efforts need to be made to investigate appropriate regeneration techniques at least for economically important millets. A large scale screening methodology has to be developed in order to determine the regeneration capacity for diverse genotypes of millets as it was investigated for rice (Dabul et al., 2009). A broad range screening made for rice set a threshold of 85% of somatic embryogenesis as an earlier indicator for efficient regeneration. Although, extensive regeneration studies were made for different millets, only limited transformation experiments were conducted to date. Hence, future research needs to develop a robust transformation protocols for each type and ecotype of millet using *Agrobacterium* method.

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**Abbreviations: 2,4-D,** (2,4-dichlorophenoxy) acetic acid; **3,6-D,** dichloromethoxybenzoic acid; **ABA,** abscisic acid; **BA,** 6-benzylaminopurine; **dicamba,** 3,6-dichloro-2-methoxybenzoic acid; **$GA_3$,** gibberellic acid; **IAA,** indole-3-acetic acid; **KIN,** kinetin; **NAA,** α-naphthaleneacetic acid; **PAA,** 2-phenylacetic acid; **pCPA,** 4-chlorophenoxyacetic acid; **picloram,** 4-Amino-3,5,6-trichloropicolinic acid; **TDZ,** thidiazuron; **TIBA,** 2,3, 5-triiodobenzoic acid.

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