

Research Article

An Effective Procedure for In Vitro Culture of *Eleusine coracana* (L.) and Its Application

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Efficient protocols for callus production, plantlet regeneration, protoplast isolation, and micronucleation of finger millet (*Eleusine coracana* (L.) Gaertn.) were developed. White nodulated calli were formed on medium with N_6 macrosalts, MS macrosalts, 2,4-dichlorophenoxyacetic acid (2 mg L^{-1}), kinetin (0.4 mg L^{-1}), 1-naphthalene acetic acid (2 mg L^{-1}), and certain additives. It was found that appropriate supplementation leads to formation of numerous shoots. Healthy rooted plantlets formed on hormone-free media. Although different tested additives had no significant effect on percentage of callus formation, it affected callus quality that further dictated plant-forming capacities. Seedlings were better source tissues for protoplasts isolation compared to callus cultures. About 5×10^6 protoplasts were isolated from one gram of seedling coleoptyles. Microcolonies were visible after 20–25 days' incubation on KM8p medium supplemented with glutamine (100 mg L^{-1}) and proline (500 mg L^{-1}). Here we also present a procedure of an efficient induction of micronuclei after chlorpropham ($10 \mu\text{M}$) and cytochalasin-B ($20 \mu\text{M}$) seedlings treatment with subsequent microprotoplasts isolation. This technique is discussed for the transfer of alien chromosomes and genes from finger millet by microprotoplast-mediated chromosome transfer.

1. Introduction

Techniques of plant biotechnology have emerged as an important aid to the traditional breeding methods for rapid genetic improvement and for integration of new genes into existing crop varieties. Success with the cell culture establishment in vitro and plant regeneration for most of the cereal and grass species has given impetus for further work with less researched crop species, especially millets which are of immense importance for solving the food and forage problems for many countries. Millets are important because they are grown in poor soils with limited inputs and they constitute a major source of food for resource-poor farmers of the areas of their cultivation [1]. The projected food demand for 2025 [2] will require the yield of millets to rise from 2.5 to 4.5 t ha^{-1} . Such yield increase could be largely achieved from improved varieties transgenically modified for biotic and abiotic stress resistance using different biotechnological methods [1].

Among various millets, finger millet *Eleusine coracana* (L.) Gaertn. is one of the well-known species for its outstanding properties as a subsistence food and forage crop [1]. Finger

millet is a major crop in the arid and semiarid regions of developing countries of Asia and Africa; more small areas of it are also present in the Americas, Oceania, and Europe. Moreover, recently the new cultivars of *E. coracana* adapted to temperate climate zone have been bred in Ukraine [3–5]. It is planned to widely use this new perspective crop as additional very productive and economically advantageous source for forage and seed production, as well as alternative grass source for bioethanol production in countries with temperate climate of Eastern Europe.

The elaboration of effective protocols for in vitro cell culture production, plant regeneration, and protoplasts isolation will be the first step towards the biotechnology for improvement of this species by genetic transformation. The previous reports on *E. coracana* dealt only with regeneration of plants through organogenesis, multiple shoot production, and somatic embryogenesis [6–9], and these data are summarized in several reviews [1, 10, 11]. The attempts also have been made to establish gene transfer system for finger millet [12–17]. Moreover, up till now there was no obvious information about isolation and regeneration of protoplasts from

finger millet that can be used for different cell and genetic engineering manipulations, including effective methods of microprotoplast-mediated chromosome transfer (MMCT) in interspecific or intergeneric breeding [18].

The present communication proposes an advanced and rapid method of plant regeneration through seedling callus cultures of *E. coracana* (L.) Gaerth. Here we also present an effective procedure of protoplast isolation and an efficient induction of micronuclei with subsequent microprotoplast isolation of finger millet. This technique is discussed for the transfer of alien chromosomes and genes from finger millet by MMCT.

2. Materials and Methods

Seeds of finger millet *Eleusine coracana* (L.) Gaerth. were surface-sterilized with 70% (v/v) alcohol for 10 min followed by 5% (v/v) sodium hypochlorite for 15 min twice. After rinsed for four times with sterile distilled water, they were used for callus induction and seedling production.

2.1. Establishment of Callus Cultures. Callus induction medium was stepwise determined in the following way: (1) basal medium selection, (2) hormonal optimization, and (3) supplements refining. Five different basal media were tested in step 1: media for callus initiation from *E. coracana* (MS macro- and microsalts, MS vitamins, 1 mg L⁻¹ 2,4-dichlorophenoxy acetic acid, and 2,4-D, 3% sucrose) [19]; medium for *Setaria italica* (N₆ macro- and microsalts, N₆ vitamins, 200 mg L⁻¹ casein hydrolysate, 2 mg L⁻¹ 2,4-D, and 3% sucrose) [20]; medium for *Sorghum bicolor* (MS macro- and microsalts, MS vitamins, 200 mg L⁻¹ glutamine, 2 mg L⁻¹ 2,4-D, and 2% sucrose) [21]; and media B and E for *E. indica* [22].

For callus induction several combinations of phytohormones of 2,4-D (1; 2; 3 mg L⁻¹), kinetin, KIN (0.2; 0.4; 0.5; 0.8; 1 mg L⁻¹), 1-naphthalene acetic acid, NAA (1; 2 mg L⁻¹), and 6-benzylaminopurine, BAP (0.2; 0.4 mg L⁻¹) supplemented to the best basal medium resulting from step 1 were tested in step 2. In step 3, glutamine (100 mg L⁻¹), proline (500 mg L⁻¹), thiamine HCl (10 mg L⁻¹), tryptophan (200 mg L⁻¹), glycine (3 mg L⁻¹), and metal salts AgNO₃ (10 mg L⁻¹) and 1 μM CuSO₄ · 5H₂O (0.249 mg L⁻¹), as the commonly used ones for cereals, in five different combinations were tested as additional additives.

All media were solidified with 0.6% agar and were adjusted to pH 5.7–5.8 prior to autoclaving. Three dishes with 30 seeds each were used in each treatment. Each experiment was repeated for three times. Cultures were incubated for three weeks in darkness at 26 ± 2°C. The effectiveness of each tested medium was evaluated based on the size and morphology (color, surface structure, water content, and tissue density) of the resultant calli. Only calli exceeded 2–3 mm and more in size were scored.

2.2. Plant Regeneration. Organogenesis from callus was induced via alteration in medium hormonal concentrations.

TABLE 1: Compositions of enzyme solutions used for *E. coracana* protoplasts isolation.

Enzymes, in %	Variants of solutions						
	1	2	3	4	5	6	7
Cellulase “Onozuka” R-10	10	2	1	1	2	2	1.5
Driselase	—	—	—	0.5	—	0.5	0.5
Pectolyase	1	0.2	1	1	0.5	1	1
Macerosyme R-10	—	—	1	0.6	1	0.5	—
Hemicellulase	—	—	0.5	0.5	0.5	0.2	0.2

However, for each callus, the supplement combination was unchanged from the step 3 of callus induction. Calli were incubated for three weeks on an MS [23] medium with 1.5 mg L⁻¹ KIN and 0.2 mg L⁻¹ NAA in light with 14 h photoperiod. The resultant shoots were grown into plantlets in a basal (hormone-free) MS medium into 100 mL tubes with perlite under light incubation. Plants with well-developed root were transferred into small pots containing sterile soil and covered with glass caps, and then they were transferred to the field after two-three weeks of growth.

2.3. Protoplast Isolation. Both callus tissues and explants of seedlings (coleoptiles and mesocotyles) from 6-day-old seedlings were used in protoplast isolation. To select the most optimal conditions the tissues were first cut and digested in seven different enzyme combination solutions (Table 1) dissolved in four different osmotic media: (1) CPW [24]; (2) medium for of isolation protoplasts of *E. indica* [22]; (3) 10x N₆ medium [25] + 80 mM KCl, 100 mM mannitol (pH 5.5); (4) 0.6 mM mannitol + 80 mM CaCl₂ (pH 5.5). Tissues were digested at 25°C for 18–24 hrs. Protoplasts were purified using the following steps.

- (1) Filter the enzyme solution into a centrifuge tube through a 60–70 μm sterile nylon mesh. The debris on the mesh was washed with 3–4 mL of washing solution (155 mM NaCl; 30 mM KCl; 5 mM CaCl₂; and 15 mM glucose) to flush down trapped protoplasts.
- (2) Protoplasts were pelleted by centrifuging at 1000 rpm for 5 min. After removing the supernatant, 2–3 mL of washing solution was added.
- (3) Carefully layer the protoplast suspension onto the top of a 3–4 mL of 20% sucrose solution in a fresh centrifuge tube.
- (4) A white band of protoplast was formed at the interface of the sucrose and washing solution layers after the tube was centrifuged at 1000 rpm for 5 min.
- (5) The protoplast band was pipetted out and diluted to 5–10 mL in volume with the washing solution for cell counting.

The purified protoplasts were cultured for 20–25 days in darkness at 25°C in a KM8p [26] medium supplemented with glutamine (100 mg L⁻¹), proline (500 mg L⁻¹), 2,4-D (0.5 mg L⁻¹), and KIN (0.2 mg L⁻¹). The resultant cell aggregates were embedded in an agar medium made of 1:1

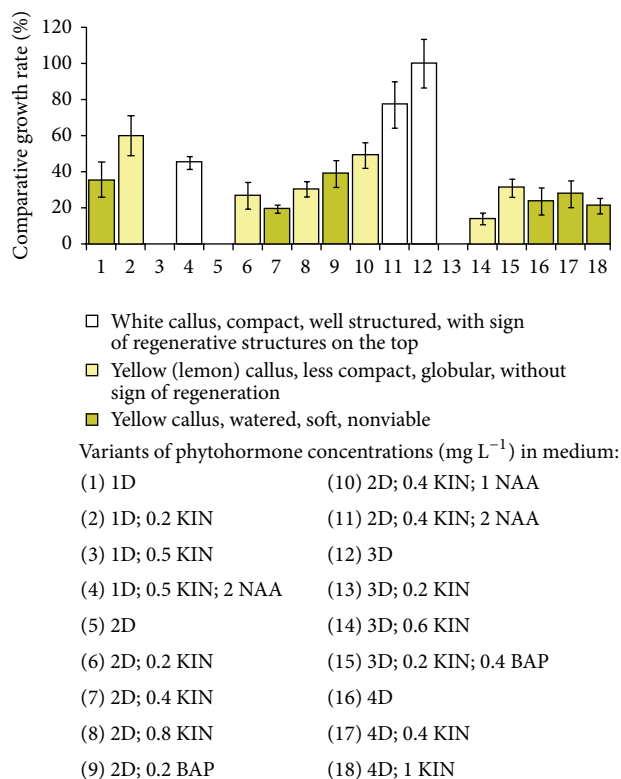


FIGURE 1: Influence of different concentrations of auxins and cytokinins on *E. coracana* callus induction.

(v/v) ratio of a KM8p medium with the above supplements to a KM8p medium supplemented with glutamine (200 mg L^{-1}), proline (1000 mg L^{-1}), tryptophan (200 mg L^{-1}), 2,4-D (2 mg L^{-1}), zeatin (0.4 mg L^{-1}), and agar (1.2%). Cultures were incubated in darkness for 3-4 days before being transferred into light for further incubation and regeneration.

2.4. Micronucleation. Micronuclei were induced by the addition of 10–150 μM isopropyl N-(3-chlorophenyl) carbamate, CIPC (chlorpropham) (Sigma, USA), and 20 μM cytochalasin B (Sigma, USA) to 50 mL of liquid MS medium with 2 mg L^{-1} 2,4-D, 0.4 mg L^{-1} KIN, and 2 mg L^{-1} NAA where 3-day-old seedlings were placed for subculture for 24 h at 25°C in the dark on a rotary shaker (120 rpm). For microprotoplasts isolation 10 μM CIPC and 20 μM cytochalasin B were added also into enzyme solution 2 (Table 1) containing 80 mM CaCl_2 and 0.6 mM mannitol as osmoticum to prevent the fusion of micronuclei and to disrupt microfilaments during protoplast isolation. The microprotoplasts were purified using the following steps: (i) we filtered the enzyme solution into a centrifuge tube through nylon mesh and washed with washing solution, as described earlier for protoplast isolation; (ii) pelleted microprotoplasts by centrifuging at 1000 rpm for 5 min; after removing the supernatant, 2–3 mL of washing solution was added; (iii) centrifuged at 1000 rpm for 5 min to collect microprotoplasts then carefully removed the supernatant and added 3 mL KM8p medium supplemented with glutamine (100 mg L^{-1}), proline (500 mg L^{-1}), 2,4-D (0.5 mg L^{-1}), and KIN (0.2 mg L^{-1}).

For cytological analysis of microprotoplasts, $3 \mu\text{g mL}^{-1}$ 4, 6-diamidino-2-phenylindole (DAPI, Sigma, USA) was added to the suspension for 10 min. The micronuclei were observed under luminescent microscope Axiostar plus (Carl Zeiss, Jena, Germany).

3. Results and Discussion

3.1. Establishment of Callus Cultures. Among the five culture media tested in step 1 of callus induction, only the Medium E supported healthy callus induction. The remaining media either failed to produce callus (Medium B) or resulted in small calli which soon became colorless and nonviable. Therefore, the basal components of Medium E [22], with N_6 macrosalts, MS macrosalts, and B5 organics [27], were chosen as the basal medium in the subsequent callus induction medium optimization tests.

Since 2,4-D is commonly considered as the most powerful callus induction hormone, it was included in all step 2 callus induction media tested. Figure 1 clearly showed that, after combined with other hormones, 2 mg L^{-1} 2,4-D resulted in the highest, up to 100%, callus formation percentages. While lower or higher 2,4-D concentrations resulted in significantly lower percentage of callus formation.

It is known that the addition of a small amount of cytokinin to the auxin-containing medium frequently inserts positive effects on callus induction. In this work, we found that the addition of an $0.2\text{--}0.6 \text{ mg L}^{-1}$ KIN always leads to a higher callus formation percentage over 2,4-D alone. Moreover KIN was also reported to have promotional effects on somatic embryogenesis of finger millet [9, 19]. To compare the effectiveness of KIN versus BAP, 0.2 mg L^{-1} KIN was replaced by the same concentration of BAP in one case, and in another case 0.6 mg L^{-1} KIN was substituted by 0.2 mg L^{-1} KIN + 0.4 mg L^{-1} BAP. The percentages of callus formation in both cases were clearly reduced. This suggested that, for *E. coracana*, KIN is a more effective cytokinin than BAP for callus induction. Similar results were reported by Pius et al. [19] for finger millet, although numerous reports indicated that BAP was preferred over KIN in induction of embryogenic calli of numerous cereal species [28].

There were three types of calli been observed in our works: (1) white, compact, well-structured callus; (2) yellow or lemon, less compact, globular callus; (3) yellow, watery, soft callus. It was found that the first type of callus (Figure 2(b)) was produced only when NAA was added to the 2,4D + KIN combinations. This type of callus was clearly superior in appearance compared to the remaining two types. It was also found that only the first type of callus stayed viable in the subsequent regeneration induction stage. Thus, despite that the NAA is not commonly used in cereal callus induction, we suggest that it is useful in light of callus regeneration capacity. This suggestion coincides with data of Kothari et al. [29] where NAA was added for plant regeneration of *E. coracana* from seeds used as initial explants.

We found also that there was no significant change in callus formation percentage regardless of the types of additional additive combinations tested supplemented to the basal media.

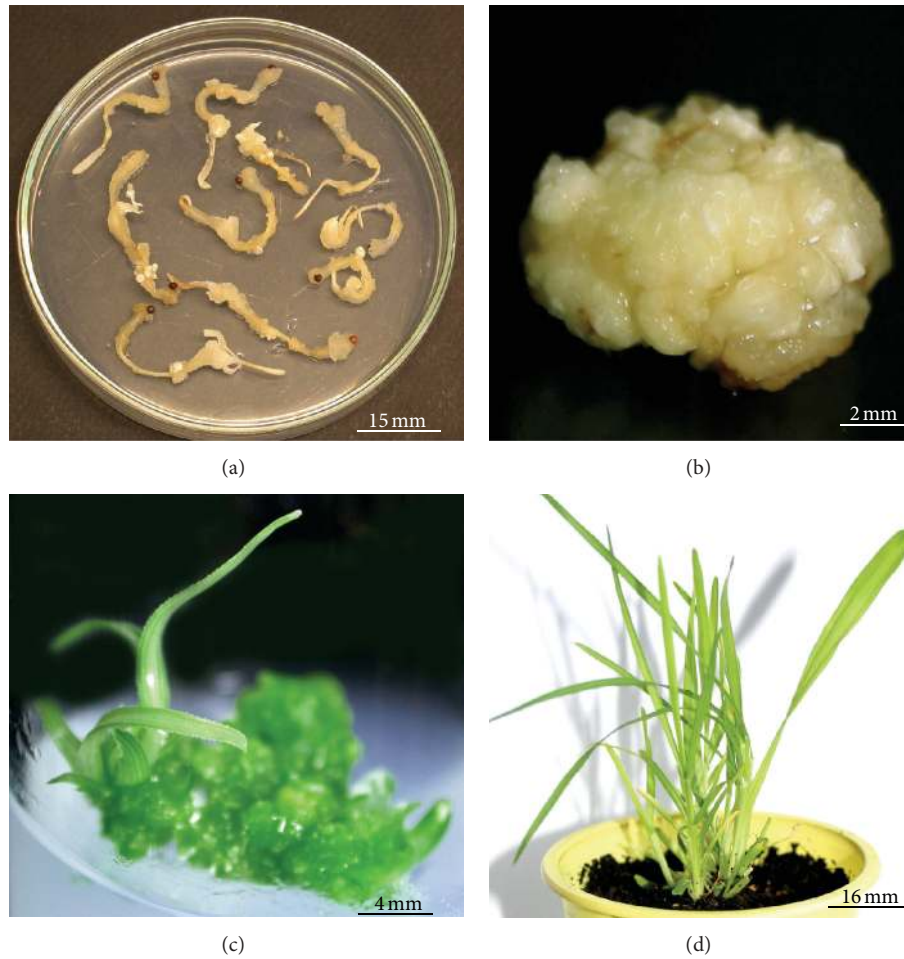


FIGURE 2: (a) Callus induction from mesocotyl of *E. coracana* from seed callus culture. (b) Morphology of compact, well-structured callus. (c) Plant regeneration of *E. coracana* from seed callus culture. (d) Rooted *E. coracana* plants in vitro.

3.2. Plant Regeneration. Although the percentage of callus formation was not affected by types of additives to the basal medium, different additive combinations greatly affected the quality of resultant tissues and resulted in significant differences in the final numbers of plantlets produced. Thus, we are presenting our results of this section in relation to the additives supplemented to the basal medium. It was found that the basal medium alone showed relatively low regeneration percentage and relatively poor tissue quality. The addition of glutamine and proline significantly improved such regeneration rate (Table 2) but failed to improve tissue quality. However, further supplementing of AgNO_3 or CuSO_4 cancelled out the advantage provided by glutamine and proline supplementation. This indicated possible toxic effects of such heavy metals.

Among the six supplementing treatments (Table 2), the supplementing of tryptophan only or supplementing of thiamine HCl and glycine in addition to glutamine and proline led to the highest percentages of regenerative calli. The resulting tissues of these two treatments appeared to have also the highest qualities and to be the most healthy. From healthy-appearing regenerated shoots (Figure 2(c)), roots developed upon transferring to hormone-free medium. Regenerating

TABLE 2: Influence of different supplements on plant regeneration of *E. coracana*.

Number	Supplements	Number of regenerants per 100 explants
1	—	0
2	100 mg L ⁻¹ glutamine 500 mg L ⁻¹ proline	30 ± 9.16
3	100 mg L ⁻¹ glutamine 500 mg L ⁻¹ proline 10 mg L ⁻¹ AgNO ₃	25 ± 8.66
4	100 mg L ⁻¹ glutamine 500 mg L ⁻¹ proline 0,249 mg L ⁻¹ CuSO ₄ · 5H ₂ O	0
5	100 mg L ⁻¹ glutamine 500 mg L ⁻¹ proline 10 mg L ⁻¹ thiamine HCl 3 mg L ⁻¹ glycine	56 ± 9.92
6	200 mg L ⁻¹ tryptophan	45 ± 9.94

* $P = 0.05$.

shoots from the nonsupplemented control treatment were of poor quality and failed to produce viable plants. The

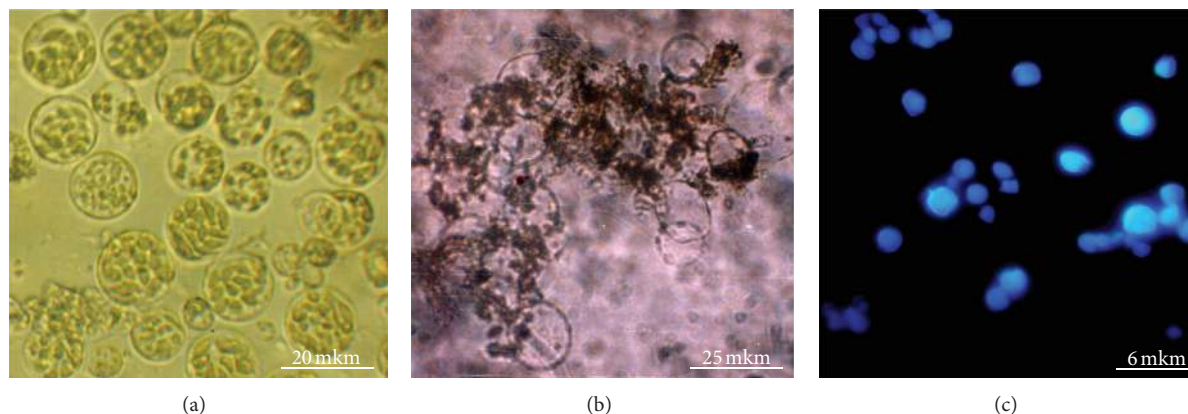


FIGURE 3: (a) *E. coracana* protoplasts isolated from seedlings. (b) Microcolonies formation in liquid KM8p medium. (c) Micronuclei in *E. coracana* cells.

supplementation of CuSO_4 inhibited root formation and did not result in plant production either. Although the supplementing of only glutamine and proline or with AgNO_3 in addition had high regeneration percentages, due to their poor tissue qualities (yellow calli with little compact structure), the number of viable plants formed was not high. In spite of their lowest regeneration percentages, treatments that led to good tissue qualities (white, well-structured calli) resulted in the highest number of viable plant production. These were treatments supplemented either with tryptophan alone or with thiamine HCl and glycine in addition to glutamine and proline.

Green and Philips [30] demonstrated that the supplementation of proline enhances somatic embryogenesis in maize, and Sirivardana and Nabros [31] showed that tryptophan favored somatic embryogenesis in some rice cultivars. Eapen and George [8], on the other hand, experienced decreased embryo germination frequency in finger millet when glutamine, proline, and tryptophan were supplemented. Thiamine was used by Chu et al. [25] to induce large quantity of cereal plant regenerants.

At the same time the supplementing of CuSO_4 did not lead to positive effect in plant regeneration. These data agreed with that of Purnhauser and Gyulai [32] that CuSO_4 supplementation inhibited rape regeneration. In contrast, Cu^{2+} enhanced rhizogenesis in barley cultures (up to 93.7%) [33], wheat, and triticale [32]. Dahleen [34] also used CuSO_4 to increase barley regeneration. The reason that led to the above discrepancy is not clear, and the role of copper in regeneration is not well understood. Since Cu^{2+} is the cofactor of many important enzymes in electron transport and protein/carbohydrate biosynthesis, Purnhauser and Gyulai [32] suggested the possibility that some of these enzymes might play certain roles in plant regeneration.

In conclusion, our results suggested that the number of plants (Figure 2(d)) eventually produced did not correlate directly with the regeneration percentage of the tissues; instead, it was closely associated with the quality of the tissues which were dictated by the hormones and supplements in the basal medium. From the obtained results, for future

experiments we propose to use the following additional composition of media for callus induction and plant regeneration of finger millet: 100 mg L^{-1} Glu, 500 mg L^{-1} Pro, 10 mg L^{-1} B_1 , and 3 mg L^{-1} Gly.

3.3. Protoplast Isolation and Culture. Among the four protoplast osmotic media, only Medium 4 supported viable protoplast isolation. Despite the CPW (Medium 1) being successful in supporting protoplast isolation for several cereal species, *Poa pratensis* [35], rice [36], and *S. bicolor* [37], its osmolarity was too low for *E. coracana* and led to the swelling of the isolated protoplasts that burst at the washing step. The same was true for Medium 3. Medium 2, on the other hand, had too high osmolarity and resulted in the condensation of all plastids at the center of the protoplasts.

Among the seven tested enzyme mixtures, Solution 2 supported the best protoplast isolation. Protoplasts resulting from this enzyme solution were spherical in shape, rich in cytoplasm, and void of large vacuoles. It was possible to reduce the time of digestion to 4 hrs.

Protoplast yield from callus was 1×10^4 protoplasts per gram of tissue; while the per-gram yield from seedling coleoptyle tissues was 5×10^6 protoplasts (Figure 3(a)). We feel that, for *E. coracana*, seedling explants resulted in sufficiently high protoplast yield upon enzyme digestion. Protoplasts started regenerating cell walls within 4-5 days in the supplemented KM8p liquid medium. Cell division took place 1-2 days later. Microcolonies (Figure 3(b)) were visible in 20-25-day-old cultures. Then they were embedded into solid KM8p medium for further plant regeneration. Thus, we described here for the first time the elaborated method of effective protoplast isolation from *E. coracana*, which could be used in future the in different programs on cellular engineering of millets, for example, through methods of symmetric or asymmetric somatic hybridization.

3.4. Micronucleation. Polygenic traits or traits with unknown biochemical or molecular mechanisms (e.g., resistance to certain diseases or stresses and other economically important traits) are still recalcitrant to transfer using methods

of genetic engineering. Recently, an alternative asymmetric somatic hybridization method using microprotoplasts (microprotoplast fusion) has been developed [18]. Since microprotoplasts contain only one or a few intact chromosomes, a limited number of chromosomes can be transferred via microprotoplast fusion, resulting in the production of chromosome addition lines with even a single and specific intact chromosome between sexually incompatible species [18, 38]. In order to apply this technique for improvement of monocot plant species, we established an efficient system for mass-preparation of microprotoplasts of *E. coracana*. Here, we present an effective method of the induction by antimetabolic agents and isolation of microprotoplast from finger millet somatic cells. As antimetabolic drugs 10–150 μM CIPC and 20 μM cytochalasin B were used for micronuclei induction in cells of finger millet seedling. Different concentration of CIPC and 20 μM cytochalasin B were added to liquid MS medium supplemented with 2 mg L^{-1} 2,4-D, 0.4 mg L^{-1} KIN, and 2 mg L^{-1} NAA where 3-day-old seedlings were placed for subcultivation for 24 h at 25°C in the dark on a rotary shaker. For microprotoplasts isolation both drugs were added also into enzyme solution to prevent the fusion of micronuclei and to disrupt microfilaments during protoplast isolation. It has been found that the most effective concentration of CIPC was 10 μM , which in combination with 20 μM cytochalasin B led to the highest percentage of micronuclei formation (Figure 3(c)). As a rule the cells with 3–4 micronuclei were observed after such treatment of finger millet cells. It is corresponding to the previously obtained data where CIPC in this concentration was successfully used to obtain microprotoplasts from developing microspores of lily species [38]. The system described here can be used for the transfer of one or a few chromosomes via microprotoplast fusion from finger millet to different economically important grasses or cereals. Because it is known that finger millet is little affected by disease and insects [39]. Chromosome addition lines produced via such technique may contribute to genetic improvement of the above-mentioned plant species.

Conflict of Interests

None of the authors has a conflict of interests with any mentioned commercial identity in the paper.

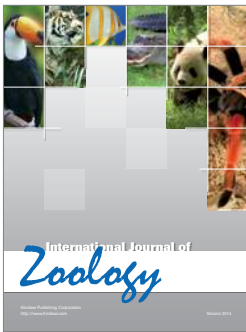
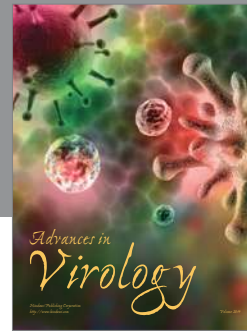
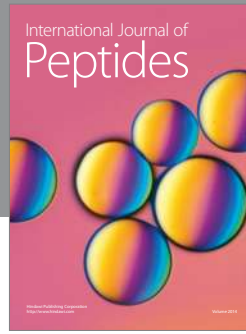
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