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Optimization of secondary hardening process of banana plantlets (*Musa paradisiaca* L. var. grand nain)

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In view of significance of well hardened planting material for ensuring highest percentage survival upon transplantation of tissue culture derived plants from shade house (secondary hardening) to farm, studies were undertaken to optimize (i) material for plantlet container, (ii) container size, (iii) potting medium, (iv) use of *Azotobacter* (for nitrogen fixation), *Aspergillus* (for phosphate solubilization) and Vesicular Arbuscular Mycorrhizae (VAM) like *G. intraradices* (for growth promotion), (v) spray of organic extracts and (vi) spacing of plantlets in shade house. These studies have revealed that (a) for the time being, there is no substitute to polybags due to susceptibility of jute bags for decay, (b) optimum size of polybag is 20×16 cm (H×W), (c) soil:press mud cake (3:1) is cost-effective and eco-friendly potting medium, (d) incorporation of *Azotobacter*, *Aspergillus* and VAMs during media preparation helps in better establishment and growth of plants, (e) sprays of organic extract have insignificant effect on the vitality of plantlets and (f) 1000 cm^2 provided optimal spacing for 3.8 plants. Financial implications of these findings are enormous.

Keywords: Banana grand nain, secondary hardening, vesicular arbuscular mycorrhizae, container size, potting media
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Introduction

It is globally recognized that tissue culture technique generates homogeneous population of plants endowed with toti-potency of elite mother plants, which are not only agro-climatically adapted, but also attributed with vibrant growth, pest resistance and consequently higher productivity. However, to achieve this objective, several problems are experienced in the growth phase (in the sterile atmosphere), primary hardening (in green house) and secondary hardening (in shade house). The first *in vitro* clonal propagation of banana was reported by Ma and Shii[1]. Improvements, practical applications and feasibility of management of tissue culture derived plants on large-scale were undertaken by Vuylsteke and De Langhe[2]. Subsequently, commercial production of micropropagated banana was established in a number of countries[3]. However, most of the R&D efforts being channeled through companies producing tissue culture plantlets of banana, information has not come into print being their guarded technological secrets. Scanty information is, therefore, available regarding national and international scenario of secondary hardening[4,5].

Problems of growth phase, primary hardening and secondary hardening need to be overcome for arriving at a commercially successful protocol of hardening[6]. Efforts were focused on optimizing parameters for secondary hardening of banana plants, Grand Nain variety after successfully standardizing the protocol for growth phase and primary hardening[7], Jalgaon District being the banana basket of India (by virtue of contributing 16% banana). Efforts made in this direction are discussed in the present article.

Materials and Methods

All secondary hardening trials were conducted at the secondary hardening station of Jain Irrigation Systems Ltd., Jalgaon (Maharashtra) during the years 2002-2004. The shade houses with 50% cutout of sunlight by shade net and raised beds were used for these trials. Tissue culture raised and primary hardened healthy plants of uniform size were used. Planting in the bags was carefully done without disturbing root-ball of the plants. All the plants were subjected to drip irrigation. Secondary hardening was monitored as a function of the following parameters.

Material of Plantlet Container

For this purpose, keeping other parameters constant, two types of bags were used, viz. (i) 150 guage black polybags (20×16 cm dimension), with

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punch size perforations and (ii) jute bags of the same size (procured from M/s Calcutta Laminating Industries, Kolkata). Each treatment consisted of 50 bags, with two replications. Survival irrigation was given as and when required, looking at the availability of soil moisture.

Container Size

Planting was done in three different size of polybags, viz. 23×20, 20×16 and 20×10 cm. All the bags were black in colour, 150-guage thick, with 25-32 punch size holes and cut at bottom edges for proper drainage. Other parameters (potting media, irrigation, fertigation and prophylactic spray schedule for pest/disease control) were kept constant. Each treatment was replicated thrice, 150 bags per replica.

Potting Media

Planting was done in the polybags filled with soil fortified with seven types of matrices, viz. press mud cake (PMC), saw dust (SD), rice husk (RH), cattle dung manure (CDM), coir pith (CP), organic manure (OM) and poultry manure (PM), each in combination with river bed soil. The control bags were filled with river bed soil only. The proportion of soil:matrix in each treatment was kept constant. Each treatment was replicated thrice, with 150 plants in each replica.

***Azotobacter, Aspergillus* and VAMs**

Polybags of 20×16 cm were filled with an optimized combination of riverbed soil and PMC. *Azotobacter* and *Aspergillus* cultures isolated from the elite banana plant and VAM, viz. *G. intraradices* (procured from TERI, New Delhi) were used. While the control plants (T0) were not treated with any of the microbes, experimental plants were treated with (i) VAM (10 g/plant, 50 spores/g) (T1), (ii) *Azotobacter* (1×10^9/mL) and *Aspergillus* (1×10^8/mL) per plant (T2) and (iii) VAM (10g/plant, 50 spores /g), *Azotobacter* (1×10^9/mL) and *Aspergillus* (1×10^8/mL) per plant (T3). All the microbes were applied to potting medium prior to planting in the polybags. Each treatment was replicated thrice, with 150 plants per replica.

Sprays with Different Organic Extracts

The treatments include sprays (on 2nd and 10th day after planting) of (i) water (control), (ii) vermiculture extract @ 0.5 mL/L (NPK 0.1% each, EC 11.82 and *p*H 8.15), (iii) organic manure extract @ 0.5 mL/L (NPK 0.15% each, EC 14.6 and *p*H 7.6), (iv) Humex @ 0.5 mL/L (Natural Products Ltd., Ahmedabad), (v) water soluble NPK 19:19:19 @ 0.5 g/L and (vi) Multiplex @ 1.5 g/L (Karnataka Agro Chemicals, Bangalore). Each dose was as recommended by the manufacturer and treatment was replicated four times, with 200 plants per replica. Other parameters like irrigation and fertigation were kept constant.

Spacing of Plantlets

Planting was done in black polybags of 20×16 cm size with a common potting mixture. The plants were kept at different spacings: 4.7 bags per 1000 cm^2 (S1), 3.8 bags per 1000 cm^2 (S2), and 2.8 bags per 1000 cm^2 (S3). All other parameters were kept constant.

All the experiments were carried out in shade house, where ambient temperature, light intensity and humidity were modified by micro sprinklers. Such modification led to reduction of (i) temperature by 2-3°C, (ii) light intensity to 50% and (iii) humidity in the increasing order as a function of secondary hardening.

Parameters Monitored

All the parameters were measured on 45th day after secondary hardening.

Plant height was measured from the base of the stem in the polybag to the angle made between the youngest and 1st open leaf.

Girth of pseudostem was measured at 5 cm from the base of the plant in the polybag.

Numbers of photosynthetically active leaves (with more than 50% of green area) were counted.

Leaf length was measured from petiole to tip and leaf width at its widest part.

Other parameters measured were number of primary roots, their maximum length, average thickness of primary roots, fresh and dry weight of whole root mat and percent mortality.

Chlorophyll was estimated as clear extract of leaves in 80% aqueous acetone as per Tandeau de Marsac and Houmard[8].

The data was analyzed statistically and tested for its significance using ANOVA software (M/S Indostat Services Ltd., Hyderabad).

Results and Discussion

The experimental strategy was to optimize only one parameter at a time (keeping other parameters constant) so that a cause and effect relationship could be established. The parameter that provided optimal result was then incorporated into experimental strategy in the next experiment. Thus, by a sequential optimization of parameters, one at a time, all the parameters were optimized one after another, so that an integrated protocol could be evolved.

Material of Plantlet Container

It was found that the growth of plants in jute bags was significantly poor as compared to the growth in polybags (Table 1). Secondly, it was observed that water requirement in jute bags was nearly double to keep plants vibrant as compared to polybags, due to more evaporation losses. Thirdly, the major drawback observed with jute bags was their susceptibility to decay within 20 d of planting due to persistent moist condition, consequently creating problems for transportation from shade house to farmer's farms. Our earlier attempts to use coarse textile bags were also in vain due to their rapid decay. Thus, poor plant growth, double requirement of scarce and vital commodity like water and problems foreseen in plantlet transportation led us to reject the use of jute bags and opt for polybags, although they are not eco-friendly and deserve replacement to meet norms laid down for ISO certification.

While each polybag costed Rs. 0.37, the cost of jute bag was Rs. 0.65. The use of polybag permitted saving of Rs. 38,000 per lac plantlets.

Table 1—Biometric parameters of banana plants as a function of container material

Container type	Height (cm)	Girth (cm)	Leaf length (cm)	Leaf width (cm)	No of leaves
Polybags	21.5	5.35	15.4	7.1	5.3
Jute bags	13.5	2.31	10.4	5.2	4.3
CD at 5%	1.12	0.17	0.43	1.13	2.48

Container Size

On the basis of 9 parameters monitored, the observations summarized in Table 2 revealed that plants performed well in bags of 23×20 cm size and were almost on par with plants in bags of 20×16 cm size. On the contrary, plants in 20×10 cm bags showed significantly poor performance in all parameters, except chlorophyll content than other two sizes. The root system in the later size bags was poorly developed as compared to first two size bags (Table 2). This indicated that the quantum of growing medium affected the root system and consequently the plant growth.

While the cost of optimally performing bags was comparable, use of 23×20 cm bags was kept at abeyance in view of their requirement of 0.65 kg more medium per polybag, in turn more cost of Rs. 65,000 per lac plantlets. Thus, by principle of exclusion, 20×16 cm bags were chosen on merit of requiring less of medium, without compromising with growth parameters.

Potting Media

The potting media are known to play an important role in the growth and development of plants in the nursery stage[9]. The control (T0, without any organic

Table 2—Biometric parameters of banana plants as a function of container size

Container size (cm)	Height (cm)	Girth (cm)	Leaf length (cm)	Leaf width (cm)	No of leaves	No of primary roots	Max.root length (cm)	Wt of roots (g)	Chlorophyll mg/g
23×20	23.4	5.33	23.1	11.53	8.7	11.3	27.35	32.5	5.57
20×16	22.9	5.27	22.6	11.18	8.4	11.2	26.42	30.6	5.42
20×10	18.7	3.51	20.0	9.42	8.2	10.6	16.60	10.2	5.42
CD at 5%	0.529	0.098	0.935	0.256	1.55	1.362	0.246	0.593	0.108

Table 3—Biometric parameters of banana plants as a function of potting medium

Potting medium	Height (cm)	Girth (cm)	Leaf length (cm)	Leaf width (cm)	No of leaves	No of primary roots	Max.root length (cm)	Wt of roots (g)	Chlorophyll mg/g
Soil alone(control)(T0)	9.8	3.52	10.9	5.1	5	7	17.9	10	4.9
Soil + PMC (T1)	17.1	5.78	19.9	9.3	8	16	38.0	30	5.3
Soil +SD (T2)	8.8	2.56	10.3	4.8	5	5	23.0	5	3.1
Soil + RH (T3)	13.6	4.63	15.3	6.8	6	8	24.5	5	5.0
Soil + CDM (T4)	11.9	4.56	13.3	5.7	6	7	19.6	10	5.2
Soil + CP (T5)	12.5	4.71	14.3	6.4	6	13	42.0	20	5.3
Soil + OM (T6)	12.4	3.30	13.8	6.2	6	10	27.6	15	5.3
Soil + PM (T7)	18.3	5.98	20.2	9.1	8	8	27.6	10	5.3
CD at 5%	0.527	0.326	0.44	0.642	1.534	1.999	1.915	0.578	0.582

PMC= Press Mud Cake; SD= Saw Dust; RH= Rice Husk; CDM= Cattle Dung Manure; CP= Coir Pith;
OM= Organic Manure; PM= Poultry Manure

matter in soil) showed poor growth of plants as compared to other treatments (Table 3). The treatment T1 (PMC) and T7 (poultry manure) offered optimal performance on par with each other in all biometric characteristics, except that root growth was significantly better in T1 as compared to T7. This is obviously due to growth promoting characteristics of PMC and poultry manure[10,11]. Eventhough the plants gave comparable performance in T3, T4, T5 and T6 with respect of shoot development, the coir pith treated plants (T5) exhibited significant root development in the polybags. This is probably on account of porous texture it imparts to the growth matrix. On the contrary, growth of sawdust (T2) treated plants seemed to be inhibited as judged from poor root, shoot, girth, leaf length/width development, including lowest chlorophyll content (Table 3). This is in accordance with the observations of Ramamurthy *et al*[12,13].

Although saw dust, rice husk and coir pith were available at paltry sum, their transportation cost rendered their use un-economical. Cattle dung manure and organic manure costed moderately; however, their composition varied as a funtion of starting material for composting. Poultry manure costed more due to limited availability and more transportation distance. In all respects, nutritive merit plus economics, PMC was preferred. On an average, PMC use permitted saving of Rs. 10,000 per lac plants.

Use of *Azotobacter, Aspergillus* and VAMs

The trial laid out to judge the efficacy of biofertilizers responded positively. Although treatment T3 performed better over all the treatments in terms of all biometric parameters, treatment T1 was also closer to it, indicating the role of VAM in the growth of plants (Table 4).

All the treatments with biofertilizers (T1 to T3) were significantly better over the control (T0). This was logical in view of complementary functions of *Azotobacter* in N_2 fixation, *Aspergillus* in phosphate solubilization and VAM in multiple functions[14]. The lowest mortality rate in VAM treated plants confirmed its importance for plant establishment during secondary hardening. Among T1, T2 and T3, superior performance was due to T3 as a result of complementarity in the functions of 3 biofertilizers. These results were in accordance with other findings[15,16].

While use of these biofertilizers added an extra cost of Rs. 10,000 per lac plantlets, reduced mortality rate by 4-5% upon transplantation in the farm more than compensated by virtue of increased sales and better image of the company product.

Sprays of Organic Extracts

Use of several foliar sprays is recommended in literature for promoting photosynthesis[17,18]. However, instead of synthetic growth promoters, natural ones are preferred for organic farming. On this background, sprays summarized in Table 5 were given. Surprisingly, none of the sprays showed significant response on the growth of plants, vis-à-vis control (with mere water spray). While this indicated no major role of these foliar sprays in the growth of plants, it is inexplicable as to why humus or Multiplex did not exert growth-promoting effect for which they are known[19]. Non-performance of humus or organic extract (aqueous extract of organic manure) in the present instance is all the more puzzling when literature is replete on their beneficial effect on the plant growth. In our opinion, the recommended dose may be for regular crop and falls short in active growth phase witnessed during secondary hardening.

Table 5—Biometric parameters of banana plants as a function of spray with organic extracts

Spraying with	Height (cm)	Girth (cm)	Leaf length (cm)	Leaf width (cm)	No of leaves
Water (control)	10.0	2.1	15.8	7.4	6
Vermiculture extract	10.2	2.3	15.8	7.3	6
Organic extract	10.1	2.3	15.9	7.3	6
Humus	10.4	2.4	16.1	7.6	6
19:19:19 + Multiplex	10.6	2.4	16.3	7.3	6
CD at 5%	0.514	0.202	0.663	0.338	1.114

Table 4—Biometric parameters of banana plants as a function of biofertilizers

Biofertlizer regime	Height (cm)	Girth (cm)	Leaf length (cm)	Leaf width (cm)	No of leaves	Mortality %	No of primary roots	Max.root length (cm)	Wt of roots (g)
T0	14.12	4.53	17.50	8.00	5.92	3.00	13.00	29.60	26.30
T1	18.00	6.05	21.40	9.30	7.00	0.89	16.00	36.10	32.10
T2	16.20	5.12	18.80	8.90	6.58	1.43	14.00	32.30	30.10
T3	18.44	6.45	22.00	9.32	7.84	0.85	18.00	38.60	34.20
CD at 5%	0.158	0.047	0.243	0.262	0.199	0.153	1.202	0.288	0.447

Table 6—Biometric parameters of banana plants as a function of spacing in shade house

Plantlet spacing per 1000 cm^2	Height (cm)	Girth (cm)	% Plants < 20 cm	% Plants > 25 cm	Plants bet. 20-25 cm	Mortality %
4.7 (S1)	24.3	15.43	18.67	26.7	54.67	0.45
3.8 (S2)	21.5	15.30	19.67	15.0	65.33	0.47
2.8 (S3)	21.0	17.46	15.33	12.0	72.67	0.16
CD at 5%	2.172	0.296	3.294	3.544	5.755	0.404

Effect of Spacing in Shade house

It is obvious from Table 6 that after 40 d, treatment S1 (4.7 plants/1000 cm^2) plants attained more height than S3 (2.8 plants/1000 cm^2), which was almost on par with S2 (3.8 plants/1000 cm^2). Whereas pseudostem girth in S1 was almost on par with S2, it was significantly less than in S3. Also number of plants between 20-25 cm was more in S3, reflecting uniform development of plants. The results indicated competition of plants for light in close spacing (S1) making them lanky and susceptible to lodging during transportation.

One acre size shade house costs about Rs. 9 lacs and permits hardening of one lac plants. Spacing in S2 thus permitted saving of about 25% space and in turn cost of Rs. 2.25 lacs per lac plantlets. Therefore, to economize the space, spacing in S2 was considered optimal.

Conclusion

In the light of these studies, an optimized protocol of secondary hardening of tissue culture derived banana plantlets comprised of (i) use of polybags for raising banana plants in shade house, (ii) bag size of 20×16 cm, which was adequate for obtaining healthy plants, (iii) press mud cake (PMC) mixed with soil (T1) as the optimal medium for producing sturdy plants, (iv) application of N_2 fixer, PO_4 solubilizer and VAM and (v) accommodation of 3.8 plants per 1000 cm^2 for getting plants of uniform size. In totality, optimized hardening protocol permitted saving of Rs. 38,000+65,000+10,000+2,25,000=Rs.3,38,000 per lac plantlets.

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