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Short Communication

In vitro pollen germination of some wild species of pigeonpea (*Cajanus cajan*) using PGM droplet technique

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Abstract

***In vitro* pollen germination is the most widely used technique for testing viability of pollen grains in a breeding programme. The germination medium (PGM) for pigeonpea species belonging to tertiary gene pool *viz., Cajanus cinereus*, *Rhyncosia rothii* and *R. aureus* has been modified from the earlier recommended medium. The improvement was done by adding poly ethylene glycol, which supported over 90% pollen germination and pollen tube growth of above mentioned species. It is expected that the use of the improved PGM will facilitate inter-specific and inter-generic hybridization imposed by biological barriers by resorting to pollen storage (in case of temporal isolation), *in vitro* fertilization and pollen selection etc. This is the first report of *in vitro* germination medium for these three tertiary gene pool species.**

Key words: *Cajanus cineres, Rhyncosia rothii, R. aureus, in vitro* pollen germination

In vitro pollen germination is the most widely used technique for testing the viability of pollen grains in breeding programs [1, 2]. The cultivated pigeonpea is endowed with a wealth of wild species in the genus *Cajanus* which includes 32 species [3]. However, only 12 wild species produced hybrids with pigeonpea [4]. Presence of strong incongruity barriers prevents the realization of hybrids with pigeonpea [5]. *In vitro* pollination/fertilization is one of the method of overcoming crossability barriers [6] for which *in vitro* pollen germination medium is a prerequisite.

The author has standardized pollen germination medium for the first time for cultivated pigeon pea [7]. Also, the pollen germination media for *Cajanus volubilis* and an improved medium for *Cajanus platycarpus* were standardized [8]. The requirements for pollen germination under *in vitro* condition would reveal information about the different constituents, temperature etc. which in turn may reflect the nutrient status of the stigmatic surface of respective species. With this rationale, this study was conducted to standardize an *in vitro* pollen germination medium for three wild pigeon pea *viz Cajanus cinereus*, *Rhyncosia rothii* and *R. aureus.*

Brewbaker and Kwack (BK) medium [9] was used (10% sucrose, 100 mg l^{-1} boric acid, 300 mg l^{-1} calcium nitrate, 200 mg l^{-1} magnesium sulphate and 100 mg l^{-1} potassium nitrate) as the base medium for pollen germination experiments. Two sets of the key media were used in our study. The Set 1 media consists of four media namely, A, B, C and D with 5%, 20%, 30% and 40% sucrose respectively in addition to the BK salts. By addition of 15% PEG 4000 to this above media, a second set of key media (Set 2) namely, E, F, G and H were developed. These eight media were initially used.

Pollen was collected and cultured following the methods described earlier [7, 10]. A minimum of 250 pollen grains were counted from 5-10 random fields.

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Similarly, 50-60 pollen tubes were measured (using an ocular micrometer) to calculate mean pollen tube length. Incubation temperature was maintained from 18 to 28^{o}C. Initially one of the key media was selected and by altering the concentration of the media constituents pollen germination was perfected for each species. The best 3 media from each treatment were selected for observation.

PGM droplet technique and pollen tube staining

In a petridish a droplet of pollen germination medium was placed using a glass rod. Thus drops of different media could be placed within few centimeter distance between them (Fig. 1A).

1-Medium A 2-Medium B 3-Medium C 4-Medium D

5-Medium E 6-Medium F 7-Medium G 8-Medium H

Further in order to have a better visualization, a drop of stain was placed carefully over the medium droplet before observation. The extra stain was removed with a piece of dried filter paper. Acetocarmine, and components of Alexander stain *viz*., Malachite green, Orange G, Aniline Blue and acid fuschin were tested.

PGM droplet technique

With this technique, a combination of 3 x 3 or 4 x 4 media were tested for effect on pollen germination *in vitro*. In a single day, sometime 64 media were tested. Use of drop of liquid medium in cavity slides often reported inconsistent results [10]. Agarified medium was then used in the humid chamber created in petriplates. In these techniques 5-10 ml of medium was used each time. Again fresh medium has to be prepared for the same combination which prevents simultaneous testing of viability of more than one species at a time. The staining of pollen and pollen tube before observation helped in effective screening of germinated and non-germinated pollen. Among the stain tested, acid fuschin (1 %) was found to be effective (Figs. 1B & C).

(A) Standardization of pollen germination medium for C. cinereus

At 24^{o}C the medium G (30% sucrose, 100 mg l^{-1}

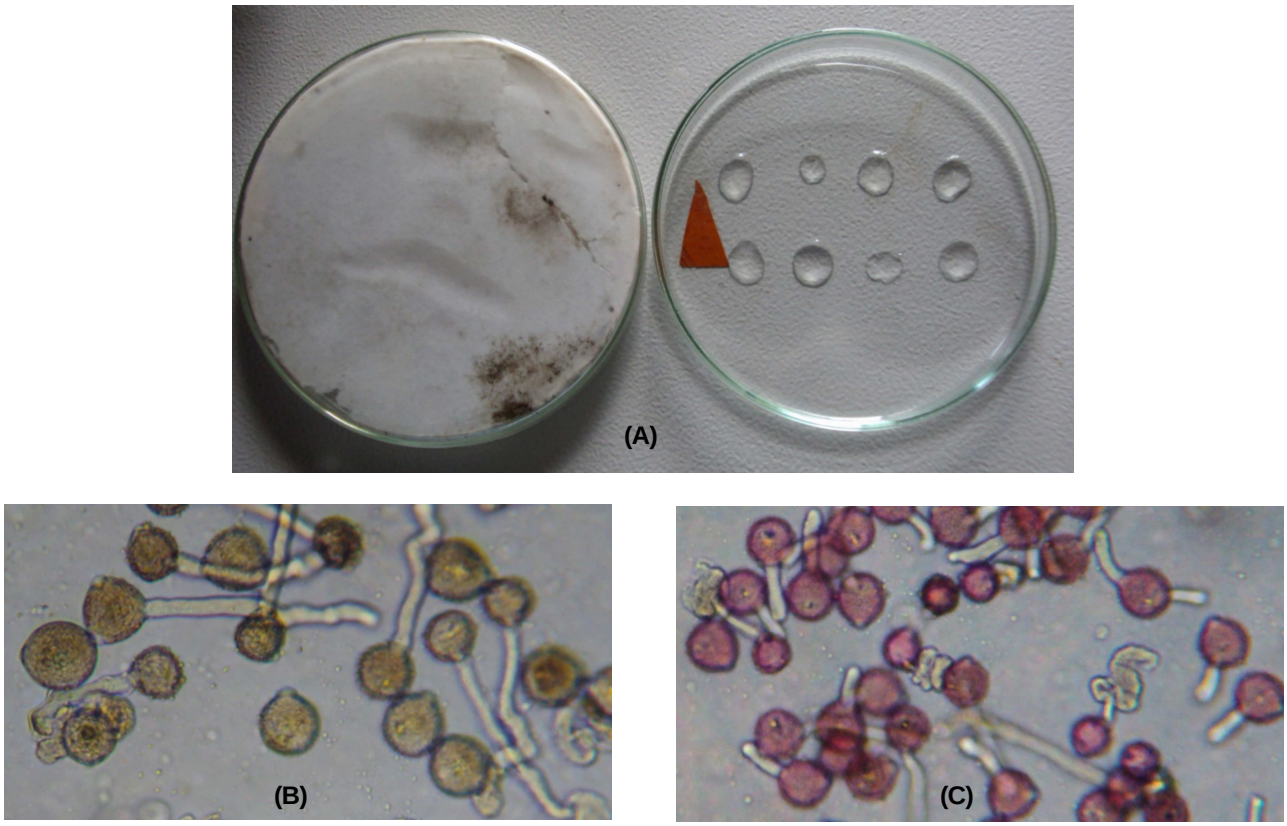

Fig. 1. PGM droplet technique (A) Incubation chamber showing 4 x 2 combination of media (B) Pollen tube without staining (C) Pollen tube with acid fuschin staining

H_3BO_3 and 300 mg l^{-1} $CaNO_3$, 15% PEG 4000 and BK) showed the maximum pollen germination, less pollen and pollen tube bursting. Hence, this key medium was selected for further modification. Different combination of the constituents (boric acid and then calcium nitrate) of medium G was attempted to improve the pollen germination (Table 1). The pollen of *C. cinereus* showed maximum germination in agar medium (GC_4) over 91 percent (Fig. 2) and mean tube length of 18.23 µm at 24°C containing 30% sucrose,

Table 1. Pollen germination (%) and pollen tube length of *C. cinereus* in various boric acid and calcium nitrate concentration

Medium	Concentration		% pollen germination	Mean pollen tube length (µm)
	Boric acid ($mg1^{-1}$)	Calcium nitrate ($mg1^{-1}$)		
G1	150	300	77.697±3.501	17±1.059
G2	200	300	77.254±3.899	18.42±1.208
G3	250	300	62.523±3.992	24.12±2.547
G21	200	100	85.705±8.875	22.09±2.465
G22	200	200	77.086±5.303	18.50±1.978
G2	200	300	76.258±2.791	16.93±2.009
GC1	225	100	91.705±8.875	21.023±2.011
GC2	225	150	87.686±5.303	17.236±2.536
GC3	225	250	76.258±2.791	17.056±2.001
GC4	225	200	91.187±1.411	20.53±1.023

225 mg l^{-1} boric acid, 200 mg l^{-1} calcium nitrate, 15 % PEG 4000 and BK salts.

(B) Standardization of pollen germination medium for Rhyncosia rothii

At 24°C, medium G showed maximum pollen germination with 66.93% and less pollen tube bursting (Fig. 3D). Among the different concentrations tried in medium G (Table 2), 200 mgl^{-1} of boric acid (G_2) supported maximum pollen germination of 73.15 with

Table 2. Pollen germination(%) and pollen tube length of *R. rothii* in various boric acid and calcium nitrate concentration

Medium	Concentration		% pollen germination	Mean pollen tube length (µm)
	Boric acid ($mg1^{-1}$)	Calcium nitrate ($mg1^{-1}$)		
G1	150	300	66.125	21.544±2.555
G2	200	300	73.152	28.870±2.352
G3	250	300	63.96	13.692±1.965
G21	200	100	86.426	23.012±3.692
G2	200	300	75.463	26.263±2.536
G23	200	200	75.896	18.023±2.012
G231	200	150	96.576	24.69±1.513
G22	200	200	81.056	18.540±3.523
G232	200	250	74.672	17.321±2.361

a mean pollen tube length of 18.87µm (Fig. 3A) and at 150 mgl^{-1} the germination improved to over 96 with reduced germination with increasing concentration .The complete medium (G_{231}) which supports over 96% germination (Fig. 3B) and maximum mean pollen tube length of 24.69µm contains 30% sucrose, 200 mg l^{-1} boric acid, 150 mg l^{-1} calcium nitrate, 15 % PEG 4000 and BK salts.

(C) Standardization of pollen germination media for Rhyncosia aureus

At 24°C after 1 hour of incubation, the maximum of over 95 per cent germination was seen in medium B with the mean pollen tube length of 42µm (Table 3).

The earliest attempt to germinate pollen of pigeonpea artificially showed 48 % pollen germination [10, 11], the liquid medium was also standardized for the wild *C. platycarpus*. A complete PGM for pigenpea genotypes was developed by supplementing the medium with PEG and e-amino caproic acid (EACA) [6]. Different molecular weight of PEG as a component of PGM was reported earlier [6, 12]. Use of EACA as

Table 3. Pollen germination percentage and pollen tube length of *R. aureus* in various media at 24°C after 1 hour

Medium	Concentration of sucrose (%)	Concentration		Percent pollen germination	Mean pollen tube length(µm)
		Boric acid ($mg1^{-1}$)	Calcium nitrate ($mg1^{-1}$)		
A	10	100	300	44.599 ± 3.560	31.462 ± 1.310
B	20	100	300	95.836 ± 2.236	42.000 ± 2.239
C	30	100	300	62.780 ± 2.012	14.692 ± 1.274

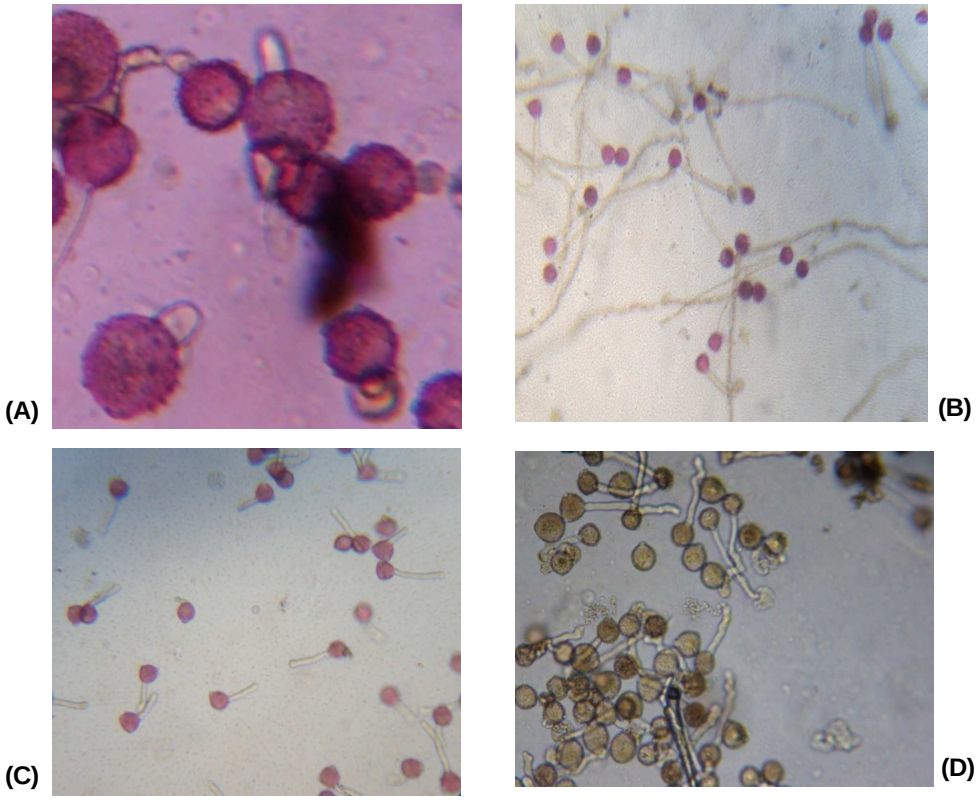

Fig. 2. Photomicrograph showing *in vitro* pollen germination of *Cajanus cinereus*. (A) pollen showing initiation of germination (B) Medium G with long irregular pollen tubes (C) Medium G with smooth pollen tubes and (D) In medium G showing pollen tube bursting at tips because of high calcium

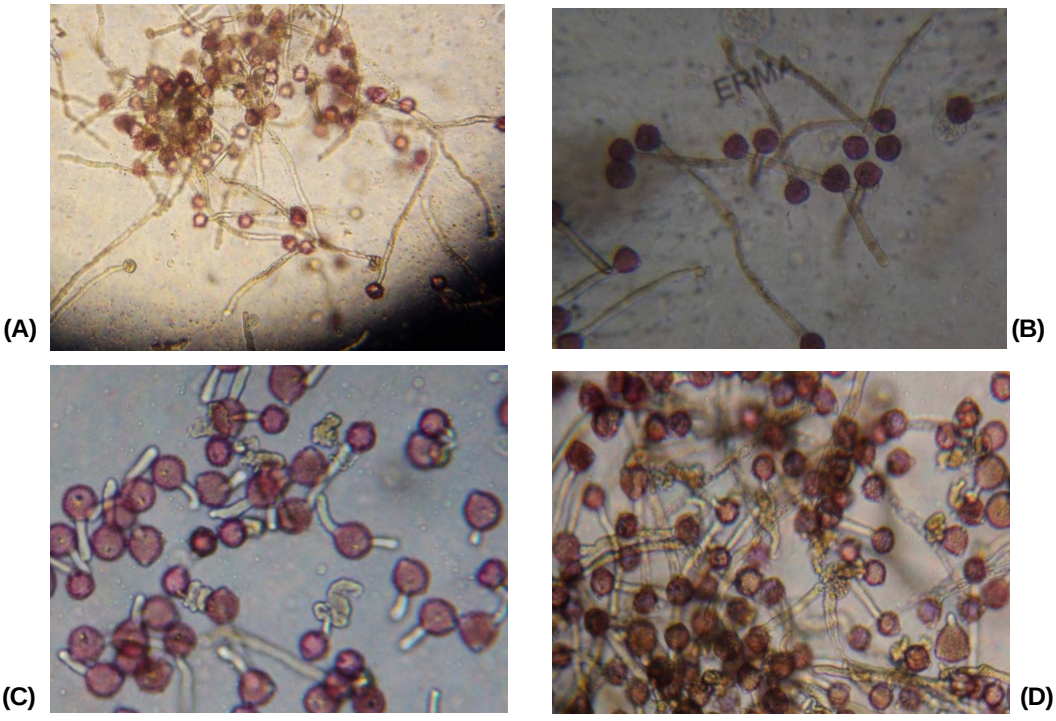

Fig. 3. Photomicrograph showing *in vitro* pollen germination of *Rhyncosia rothii.* (A) Medium G with long irregular pollen tubes(low boric acid) (B) Medium G with smooth pollen tubes (C) Pollen tip bursting in medium G with high calcium (D) Medium B with smooth pollen tube

one of the medium constituents was reported *for the first time* in 2001 by Jayprakash and Sarla [7]. They reported that different genotypes of pigeonpea differ in their requirement of constituents for effective pollen germination.

There exists lot of intraspecific variability in the whole gene pool of pigeonpea. This PGM may be used in variety of ways (1) the variability in each species may be explored (2) it may help in undertaking pollen selection experiments (3) it may help in attempting *in vitro* pollination/fertilization and (4) intra specific variability based on pollen germination medium may help in picking up appropriate accession for hybridization.

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