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Tissue culture of *Cyamopsis tetragonoloba* L. (Guar) genotypes

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Abstract
The study aimed to work out a protocol for callus initiation from five genotypes of *Cyamopsis tetragonoloba* L. plant (Gm2, Gm5, Gm6, Gm8 and Gm34). Murashige and Skoog (MS) media supplemented with 3% sucrose were used as media for culture initiation. The MS medium was supplemented with different combinations of growth regulators (BAP, IBA, NAA and 2,4-D) at one concentration. Three type of explant used were; radicle, hypocotyl and cotyledonary leaf. Explants of five genotypes of *C. tetragonoloba* were cultured in Murashige and Skoog media supplemented with different growth regulators. Results show that M2 (with NAA) failed to form callus for the five genotypes, while M1 (with IBA) and M3 (with 2-4, D) initiated callus for the five genotypes. M3 was better than M1 for callus formation for the *C. tetragonoloba* genotypes. It was concluded that the five genotypes of *C. tetragonoloba* have responded differently to the plant growth regulators.

Keywords: *C. tetragonoloba*-growth regulators- tissue culture

1. Introduction
C. tetragonoloba is a crop having unique ability to grow under harsh climate. The crop has enormous industrial utility due to the presence of gum in its endosperm which has unique biochemical properties. Due to these properties, it is used in a wide range of products varying from ice-creams to explosives. Tissue culture being a prerequisite for experiments for hybridization and improvement through genetic engineering. The effect of various growth regulators and their combinations on different explants in diverse varieties has been studied by Bansal *et al.*, 1994 [3], Ahmad and Anis, 2007 [1], Bhansali, 2011 [4], Gargi *et al.*, 2012 [7], Ahlawat *et al.*, 2013 [2] and Deepika *et al.*, 2014 [5]. Gargi *et al.* (2012) [7] had conducted the study to explore regenerative ability, appropriate media and explants of *C. tetragonoloba*. Ahlawat *et al.* (2013) [2] had studied the *in vitro* callus formation in cultivated and wild species of *Cyamopsis* in India. They found that 2mg/l- 2,4-D and 2mg/l-BAP induced callusing from cotyledons in *C. serrata* and *C. senegalensis* while *C. tetragonoloba* showed poor callus formation on the same medium. The callus proliferated well on MS medium adjuncted with 2mg/l-NAA and 2mg/l-BAP. Hypocotyl of all tested species of *Cyamopsis* showed very good callus induction response in the medium supplemented with 2mg/l 2, 4-D. As the concentration of BAP increased from 1mg/l to 2mg/l in combination with 2mg/l NAA callus formation was also increased.

2. Materials and Methods
2.1. Plant materials
Mature seeds of five genotypes of *Cyamopsis tetragonoloba* (Gm2, Gm5, Gm6, Gm8 and Gm34) were used for experimentations (El-Tahir 2017) [6].

2.1.1. Chemicals
Tissue culture media: Murashige and Skoog (MS) medium and plant growth regulators; the auxins Indole-butyric acid (IBA), α- naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) and the cytokinin, 6-Benzyl aminopurine (BAP). In addition to NaOH and HCl as general reagents**.**

2.2. Methods
2.2.1. Germination of seeds under aseptic conditions
2.2.1.1. Surface Sterilization of Seeds
Seeds of *C. tetragonoloba* from the five genotypes were selected carefully i.e. seeds with normal shape, regular size and colour were used. Abortive and damaged seeds were discarded. The surface sterilization of seeds was accomplished with an aqueous solution of sodium hypochlorite (NaOCl) at 1% concentration or by using the commercial house hold bleach "Clorox". One volume of "Clorox" was taken and 9 volumes of distilled water were added to "Clorox" solution. The selected seeds were first washed with 70% ethanol for one minute then immersed in the "Clorox" solution added to it few drops of liquid detergent for 10 minutes with occasional stirring. After the prescribed period of time, the seeds were rinsed 3-4 times with autoclaved distilled water to remove the sterilant. The surface sterilized seeds were soaked in sterile distilled water under aseptic condition for a period of 3 – 4 hours. The imbibed seeds were transferred to sterile 9 cm Petri dishes containing sterilized agar (6.4 g/l). The Petri-dishes were incubated at 30ºC in the dark for one week, after which germination percentage was calculated for each genotype.

2.2.1.2. Callus initiation
2.2.1.2.1. Culture media
The media contained Murashige and Skoog (MS) basal mineral nutrients (Murashige and Skoog, 1962) [8] supplemented with 3% sucrose were used as media for culture initiation (Table 2). For callus initiation the basal

MS media were manipulated with auxins IBA, NAA, and 2,4- D; and the cytokinin BAP in one concentration of 0.5 mg/l has been used for all types of explants. The pH of the media was adjusted to 5.5 using either 0.1N HCl or 0.1N NaOH. For preparation of semi-solid media, 0.8% agar was used as the gelling agent. The sterilization of media and glassware was carried out in an autoclave at 121°C, (15 lb/in2) pressure for 15 minutes before dispensing in culture vessels.

2.2.1.2.2. Culture vessels

Petri-dishes (plastic, ready sterile, 9cm in diameter), culture jars (200ml capacity) were used as culture vessels. Treatments with growth regulators: MS media were supplemented with various auxins (IBA, NAA, and 2,4- D); and the cytokinin BAP as shown in Table 1.

2.2.1.3. Preparation of explants

C. tetragonoloba seedlings germinated under aseptic conditions were used as a source of explants. Three types of explants were used: root, hypocotyl and cotyledonary leaves explants were excised from 7 days old seedlings and used for callus initiation on solidified MS basal medium.

2.2.1.4. Callus multiplication and maintenance

For callus multiplication and maintenance the MS medium was supplemented with different combinations of growth regulators i.e. BAP, IBA, NAA, 2,4-D at one concentration as shown in Table 1, in the presence of 3% sucrose and 0.8% agar.

Table 1: MS media supplemented with different combinations of growth regulators used for callus initiation and maintenance:

Medium code	Growth regulators combinations
Medium 1	1mg/l IBA + 1mg/l BAP
Medium 2	1mg/ l NAA + 1mg/l BAP
Medium 3	1mg/l 2,4-D + 1mg/l BAP

*Cytokinin: Benzylaminopurine (BAP), *Auxins: Indole-3-butyric acid (IBA), Naphthaleneacetic acid (NAA), 2, 4- Dichlorophenoxyacetic acid (2,4-D).

Table 2: Chemical composition of Murashige and Skoog medium (After Murashige and Skoog, 1962) [8]

Components	Mg/l
MgSO4.7H2O	370
$CaCl_2$. $2H_2O$	440
KNO_3	1,900
NH_4NO_3	1,650
KH_2PO4	170
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
MnSO4.4H2O	22.3
$ZnSO_4.7H_2O$	8.6
CuSO4.5H2O	0.025
CoCl2.6H2O	0.025
KI	0.83
H_3BO_3	6.2
$Na_2MO_4.2H_2O$	0.25
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1-1.0
Glycine	2
Sucrose	30,000.00
L-Ascorbic Acid	100
Agar	8,000.00

pH= 5.5

3. Results and Discussion

The seeds of five genotypes of *C. tetragonoloba* (Gm2, Gm5, Gm6, Gm8 and Gm34) were germinated under aseptic conditions. Table 3 Fig. 1 shows that the germinability of the seeds in all genotypes under investigation varies between 86.6-100%.

3.1. Callus initiation

Explants from the five genotypes were removed under aseptic conditions and transferred to MS medium supplemented with 3% sucrose, 0.8% agar to solidify the medium, and different types of auxins. M1 was supplemented with IBA, M2 was supplemented with NAA and M3 contained 2,4-D. All media were supplemented with 1mg/l BAP.

The results presented in Table 4 show that M1 (IBA) and M3 (2,4- D) were effective in callus initiation in the five genotypes. M3 gave better frequency than M1. M2 (NAA) failed to initiate callus in the explants of the five genotypes. M3 resulted in a good amount of callus tissues from the cotyledonary explants of genotypes Gm2 and Gm8, and from the hypocotyl explants of genotype Gm5, and to the root explants of genotypes Gm5 and Gm34. IBA in M1 when combined with 1mg/l BAP gave a good amount of callus tissues from the hypocotyl explants of genotypes Gm6, Gm8 and Gm34. Ahlawat *et al.* (2013) [2] found that 2mg/l- 2,4-D and 2mg/l-BAP induced callusing from cotyledons in *C. serrata* and *C. senegalensis* while *C. tetragonoloba* showed poor callus formation on the same medium. The callus proliferated well on MS medium adjuncted with 2mg/l-NAA and 2mg/l-BAP. Hypocotyl tissues of all tested species of *Cyamopsis* showed very good callus induction response in the medium supplemented with 2mg/l 2, 4-D.

Table 3: The Germinability of *C. tetragonoloba* seeds of five genotypes grown under aseptic conditions

Genotypes	Germination ratio %
Gm2	88.2
Gm5	86.7
Gm6	100
Gm8	86.6
Gm34	100

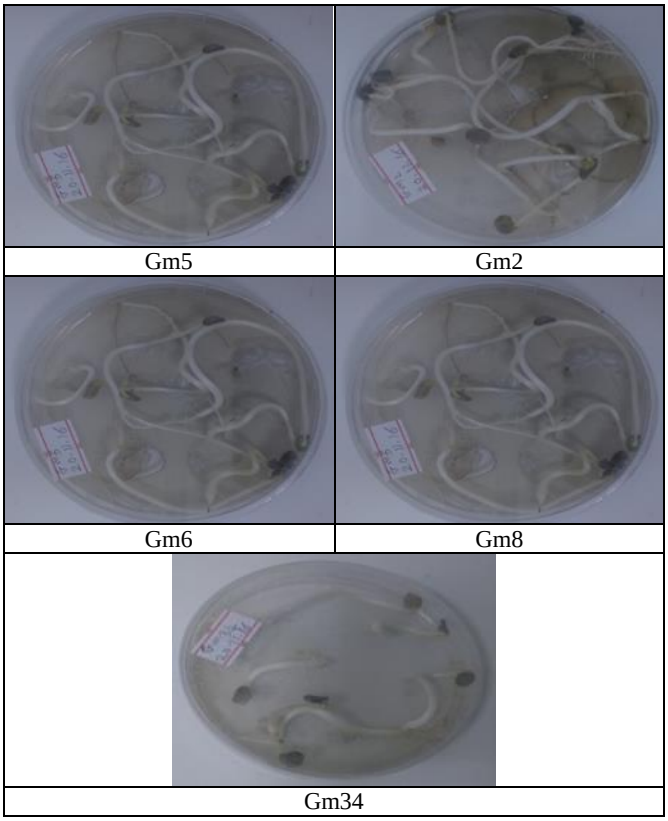


Fig 1: Seedlings from five genotypes of *C. tetragonoloba* grown under aseptic conditions and used as source of explants. 7 days old

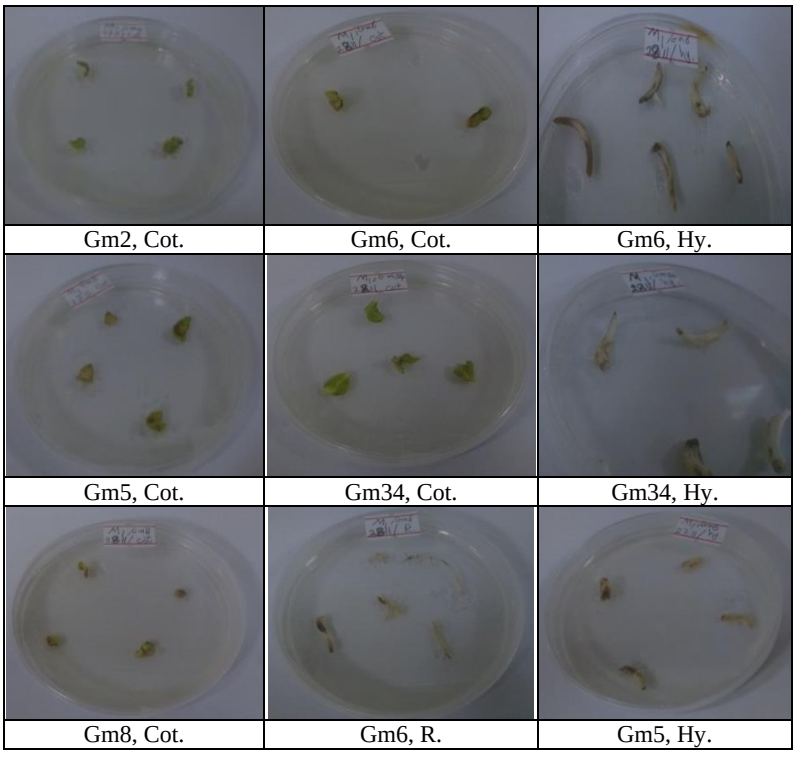


Fig 2: Callus initiation from different explants of *C. tetragonoloba* genotypes- 7 days after culturing on M1 medium Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

Table 4: Responses of explants of five genotypes to three types of media.

Genotypes	Media / Explants	M 1 (1mg/l-IBA+ 1mg/l-BAP)	M 2 (1mg/l-NAA+ 1mg/l-BAP)	M 3 (1mg/l-2,4-D+ 1mg/l-BAP)
Gm2	Cotyledonary leaves	+	-	+++
	Hypocotyl	+	-	++
	Root	+	-	+
Gm5	Cotyledonary leaves	+	-	++

	Hypocotyl	++	-	+++
	Root	+	-	+++
Gm6	Cotyledonary leaves	+	-	++
	Hypocotyl	+++	-	++
	Root	+	-	++
Gm8	Cotyledonary leaves	+	-	+++
	Hypocotyl	+++	-	-
	Root	+	-	+
Gm34	Cotyledonary leaves	+	-	+
	Hypocotyl	+++	-	++
	Root	+	-	+++

(-) = No callusing, (+) = Low amount of callus formation, (++) = Medium amount of callus formation, (+++) = Good amount of callus formation

Gm6, Cot. | Gm6, R. | Gm5, Cot.

Fig 3: Callus initiation from two explants of *C. tetragonoloba* genotypes- 7 days after culturing on M3 medium Cot.= cotyledonary leaves, R.= root

Table 5: Frequency of callus formation (%) on explants of five genotypes of *C. tetrtagonoloba* grown on three types of media

Genotype	Media / Explants	M 1	M2	M 3
Gm2	Cotyledonary leaves	100	-	100
	Hypocotyl	50	-	25
	Root	25	-	25
Gm5	Cotyledonary leaves	100	-	100
	Hypocotyl	75	-	66.7
	Root	50	-	50
Gm6	Cotyledonary leaves	100	-	100
	Hypocotyl	80	-	75
	Root	25	-	50
Gm8	Cotyledonary leaves	100	-	100
	Hypocotyl	50	-	-
	Root	33.3	-	75
Gm34	Cotyledonary leaves	100	-	100
	Hypocotyl	100	-	100
	Root	100	-	100

(-) = No callusing

Results in Table 5 show that M1 and M2 gave a percentage of 100% for all cotyledonary leaves of the five genotypes, also for the three types of explants of genotype Gm34. In M1 medium the frequency was more than 50%, just roots of genotypes Gm2, Gm6 and Gm8. Similar observation was recorded for M3 medium, while hypocotyl and root of Gm2 showed less than 50% initiation rate. Results in Table 6 show that the fastest date of initiation was 6 days in the three explants of genotypes Gm6 and Gm34 in M1, and the last one was 30 days in the hypocotyl of Gm6 in M3

3.2. Organogenesis

Organogenesis for explants in Table 7 show that M1 and M3 form a callus in the five genotypes of *C. tetrtagonoloba*, while M2 failed to form a callus for the five genotypes as shown in Fig. 11. Callus tissues initiated on M1 formed adventious roots in hypocotyl callus of genotypes Gm6, Gm8 and Gm34.

3.2.1. Callus morphology

Fig. 8, 9 and 10 show callus with creamy colour for the five genotypes. Callus initiated in M3 medium from hypocotyl of *C. tetragonoloba* two genotypes of Gm2 and Gm8, while Gm2 formed callus in M1 medium better than Gm8 as shown in Fig. 12. The same pattern observed when culturing seeds of the same two genotypes as shown in Fig. 13.

3.2.2. The effect of IBA and 2, 4-D

Observations during the experiment indicated that IBA and 2,4-D reduced linear growth of roots and hypocotyl in the early stages. It was noted that 1mg/l of IBA and 2,4-D combined with 1mg/l BAP caused a swelling and splitting of the cortical tissues throughout the length of the hypocotyl. Proliferation of cortical cells resulted in a mass of callus tissues at the lower end of the hypocotyl (Fig. 13) the growth of the radicle was highly retarded.

It was concluded that Gm2 explants and seedlings grown on M1 (IBA) and M3 (2, 4-D) initiated calluses. The callus growth was very slow and it doubled its size after 70 days. Some explants initiated adventitious roots especially the hypocotyl explants. And no shoot regeneration was observed.

Table 6: Date of callus initiation of explants of five genotypes of *C. tetrtagonoloba* in three types of media

		Days for initiation		
Genotypes	**Media** / **Explants**	**M 1**	**M2**	**M 3**
Gm2	Cotyledonary leaves	7	-	12
	Hypocotyl	7	-	21
	Root	14	-	7
Gm5	Cotyledonary leaves	7	-	7
	Hypocotyl	7	-	7
	Root	14	-	9
Gm6	Cotyledonary leaves	6	-	6
	Hypocotyl	6	-	30
	Root	6	-	6
Gm8	Cotyledonary leaves	6	-	6
	Hypocotyl	7	-	-
	Root	7	-	10
Gm34	Cotyledonary leaves	6	-	6
	Hypocotyl	6	-	6
	Root	6	-	21

(-) = no callusing

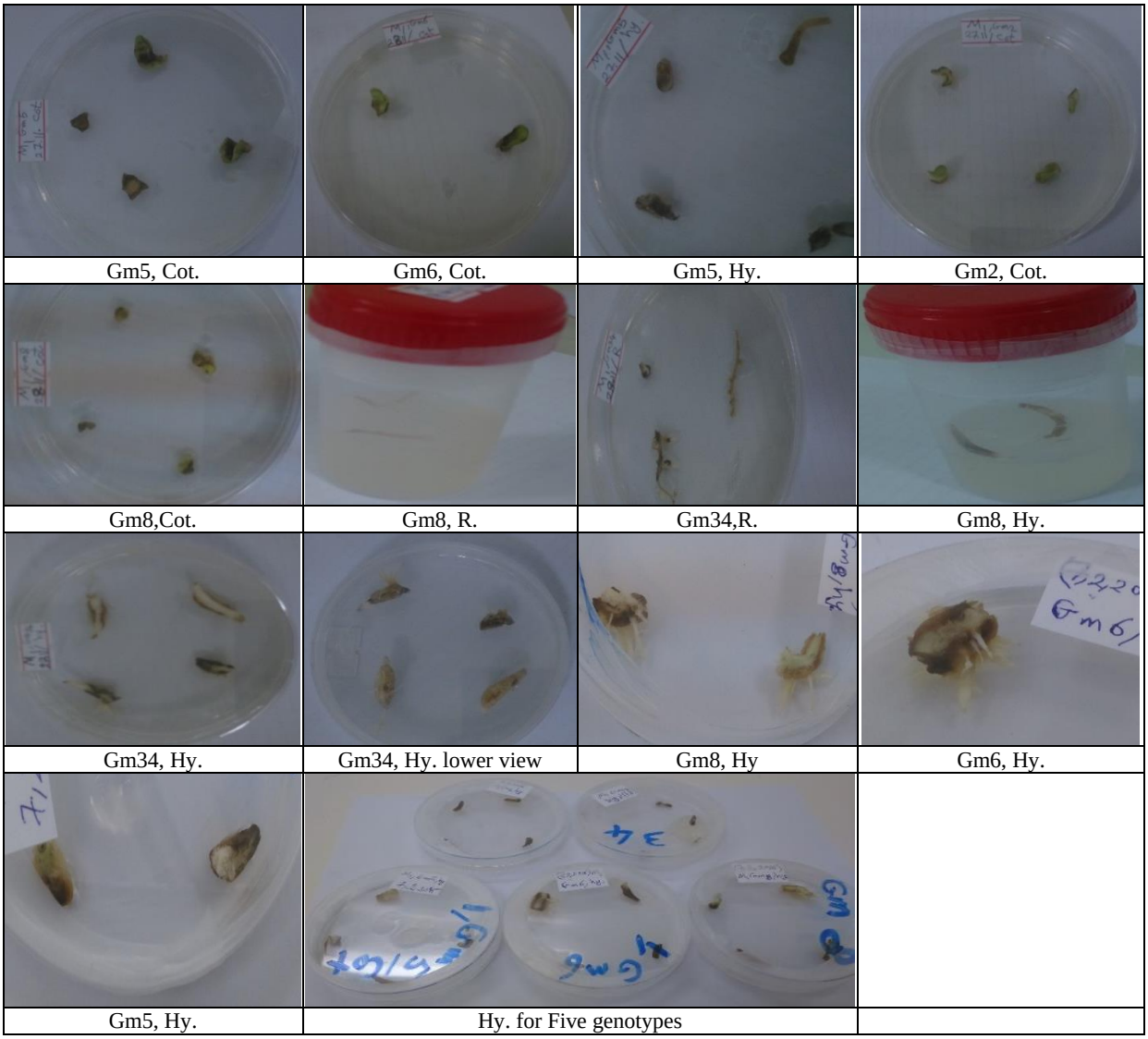


Fig 4: Callus initiation from different explants of *C. tetragonoloba* genotypes- 14 days after culturing on M1 medium. Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

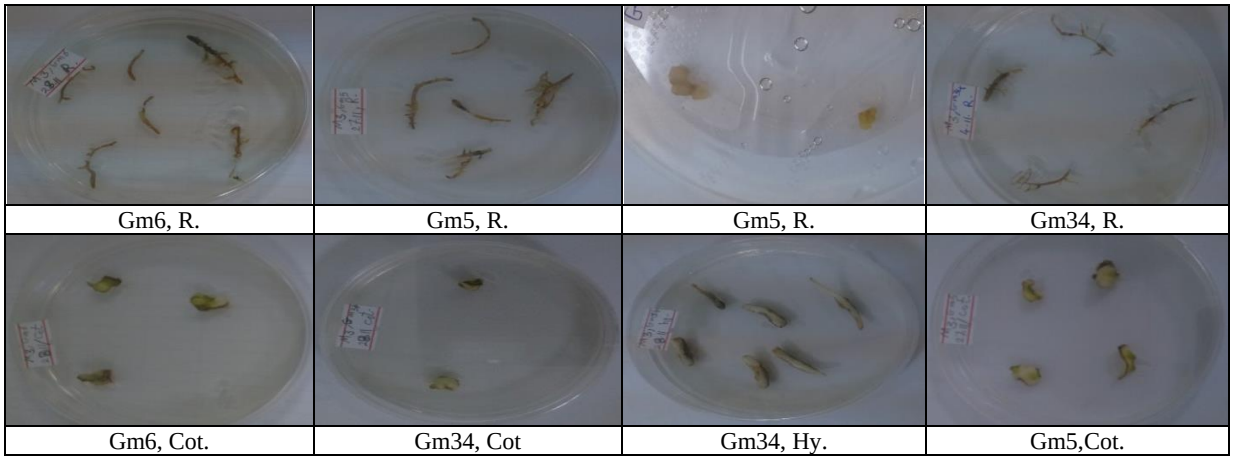


Fig 5: Callus initiation from different explants of *C. tetragonoloba* genotypes- 14 days after culturing on M3 medium.

Table 7: Organogenesis for explants of five genotypes of *C. tetrtagonoloba* in three types of media Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

	Media	**M 1**	**M2**	**M 3**
Genotypes	explant			
Gm2	Cotyledonary leaves	Callus	-	callus
	Hypocotyl	Callus	-	Callus
	Root	Callus	-	Callus
Gm5	Cotyledonary leaves	Callus	-	callus
	Hypocotyl	Callus	-	Callus
	Root	Callus	-	Callus
Gm6	Cotyledonary leaves	Callus	-	callus
	Hypocotyl	Callus + Adventious roots	-	Callus
	Root	Callus	-	Callus
Gm8	Cotyledonary leaves	Callus + Adventious roots	-	Callus
	Hypocotyl	Callus	-	-
	Root	-	-	Callus
Gm34	Cotyledonary leaves	Callus	-	Callus
	Hypocotyl	Callus + Adventious roots	-	callus
	Root	Callus	-	Callus

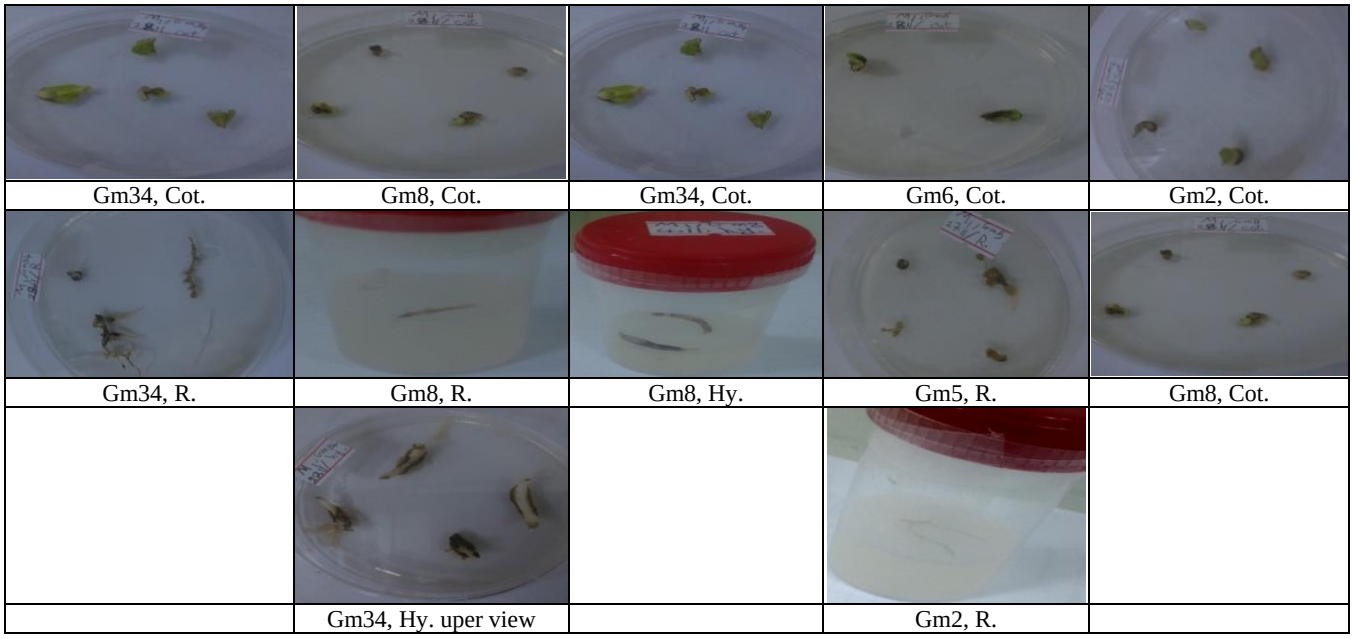


Fig 6: Callus initiation from different explants of *C. tetragonoloba* genotypes- 21 days after culturing on M1 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

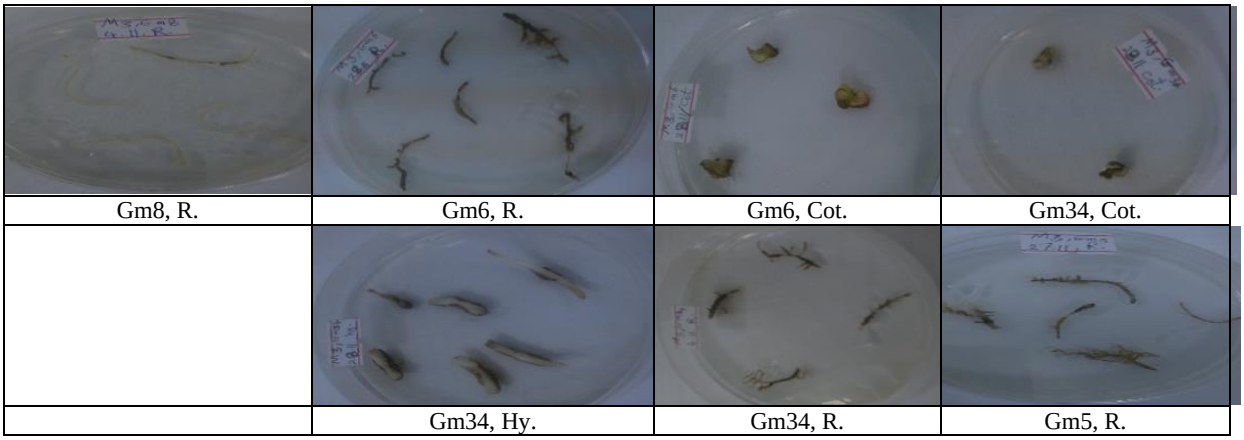


Fig 7: Callus initiation from different explants of *C. tetragonoloba* genotypes- 21 days after culturing on M3 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

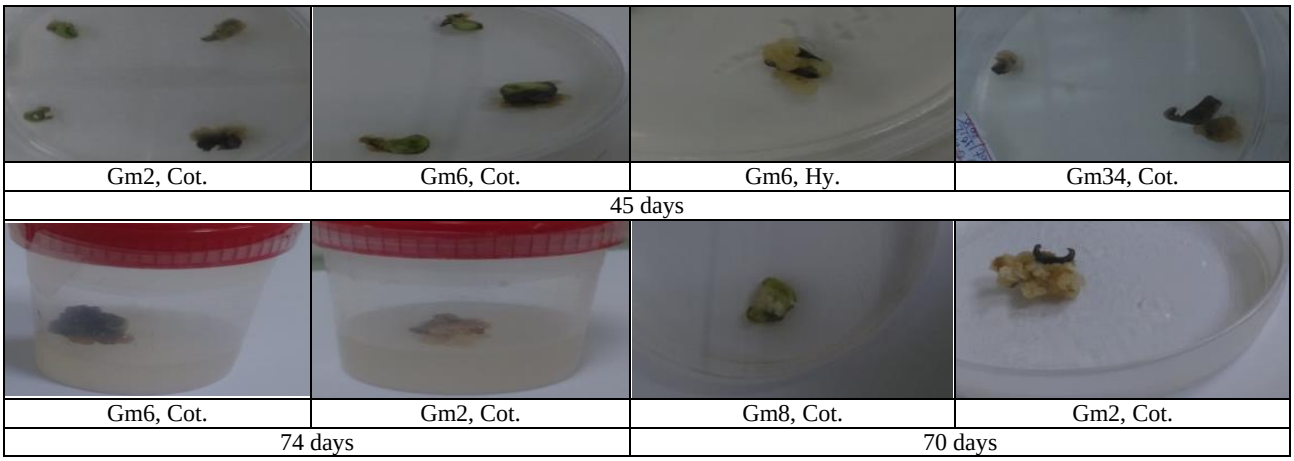
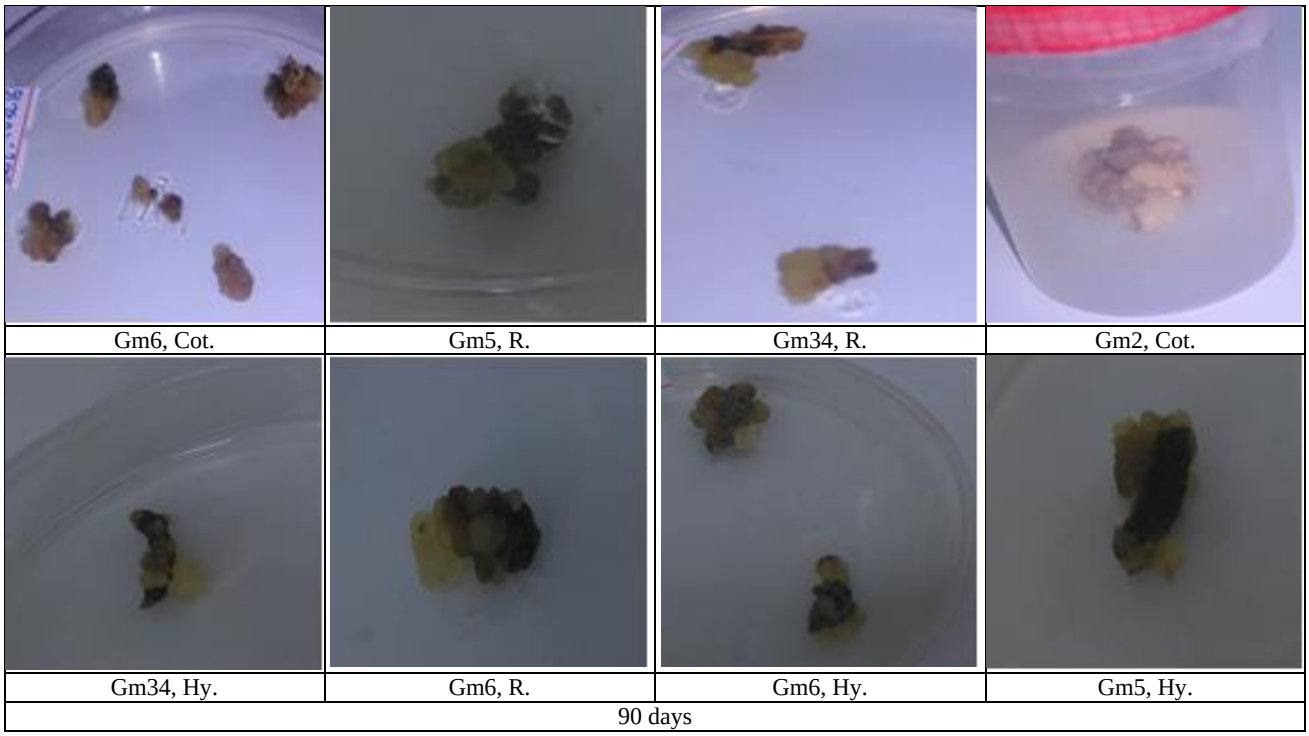


Fig 8: Callus initiation from two explants of *C. tetragonoloba* genotypes- 45, 70 and 74 days after culturing on M3 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl.



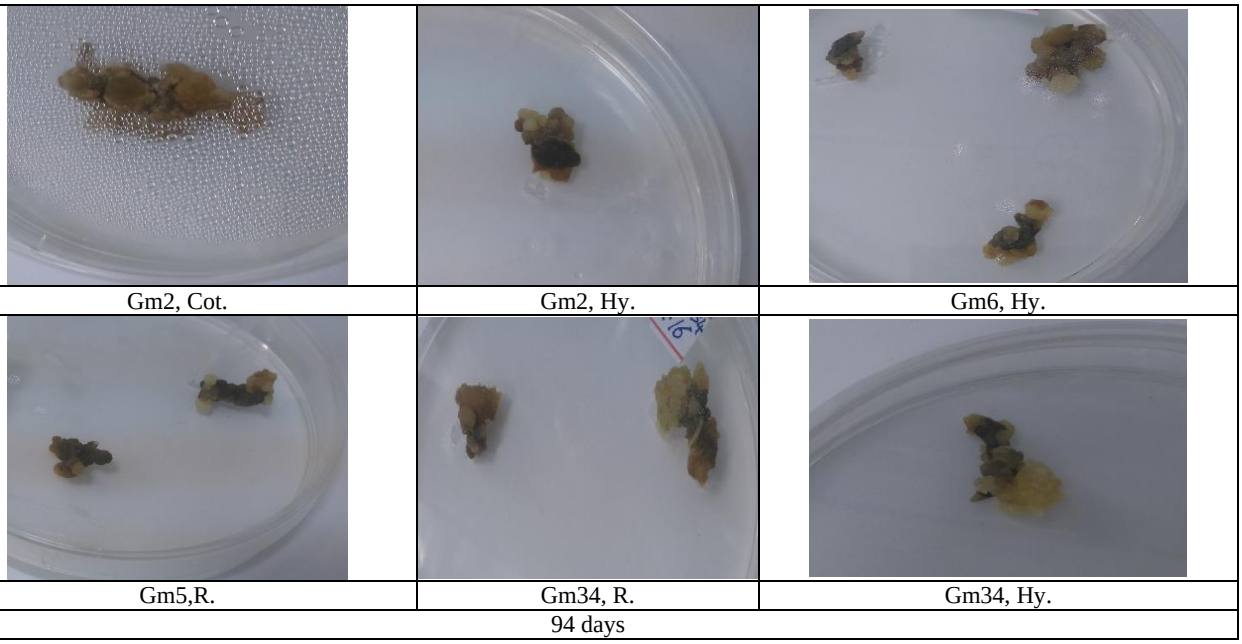
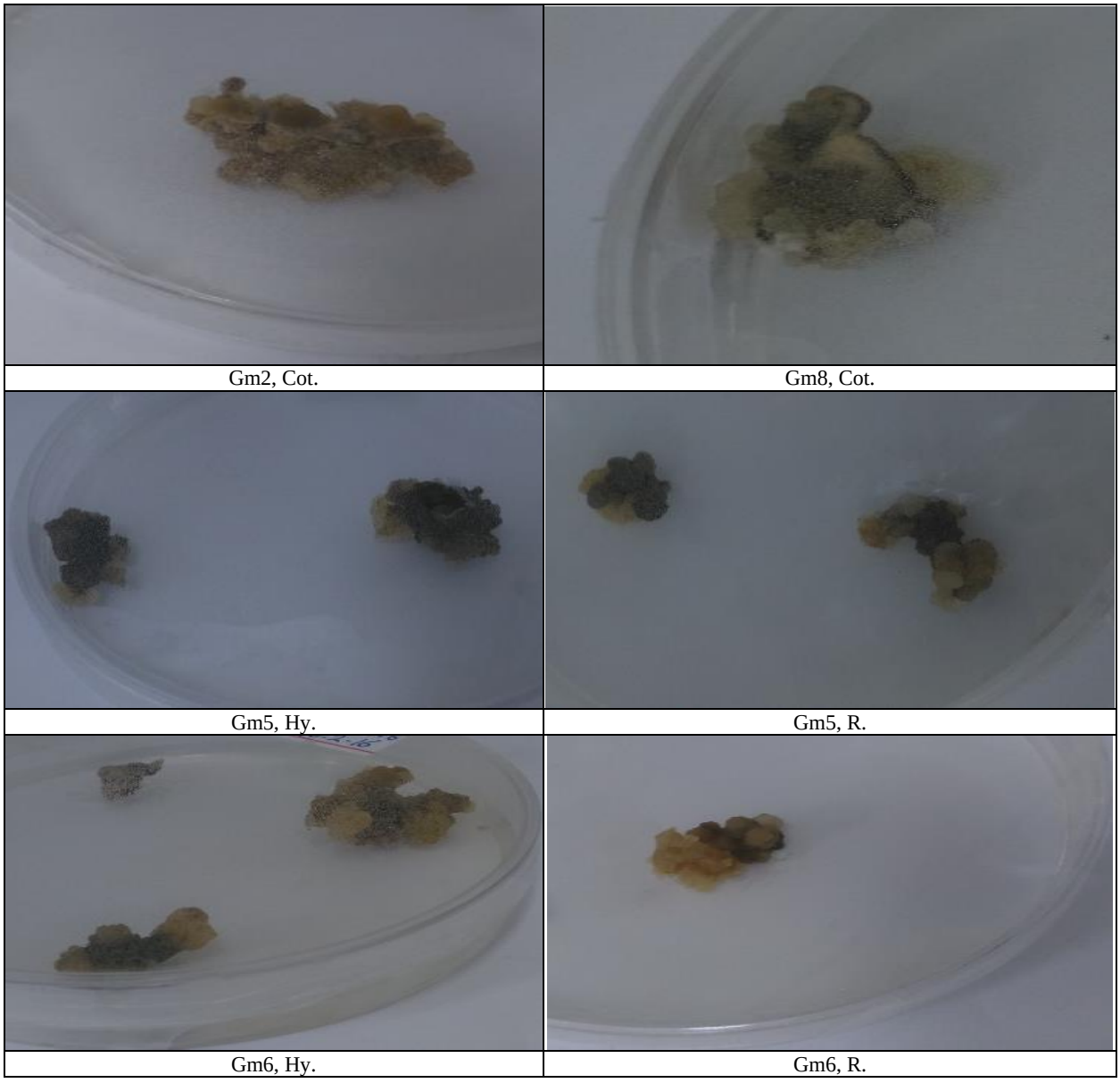
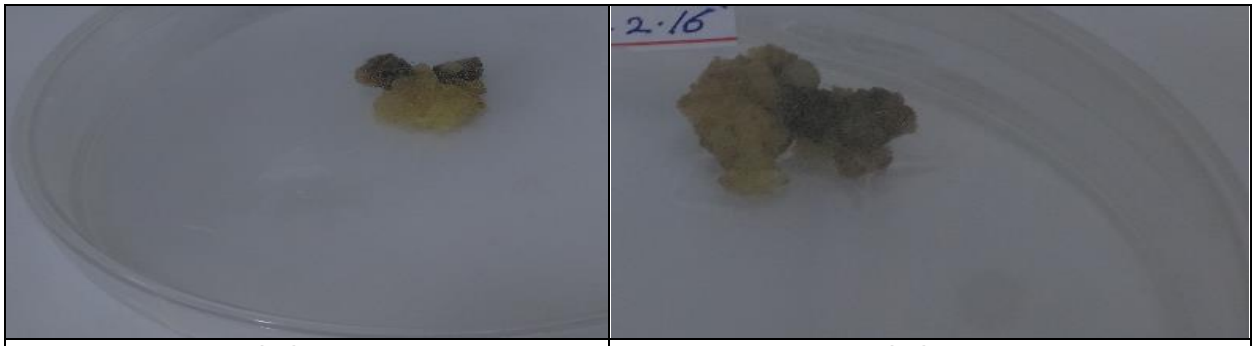


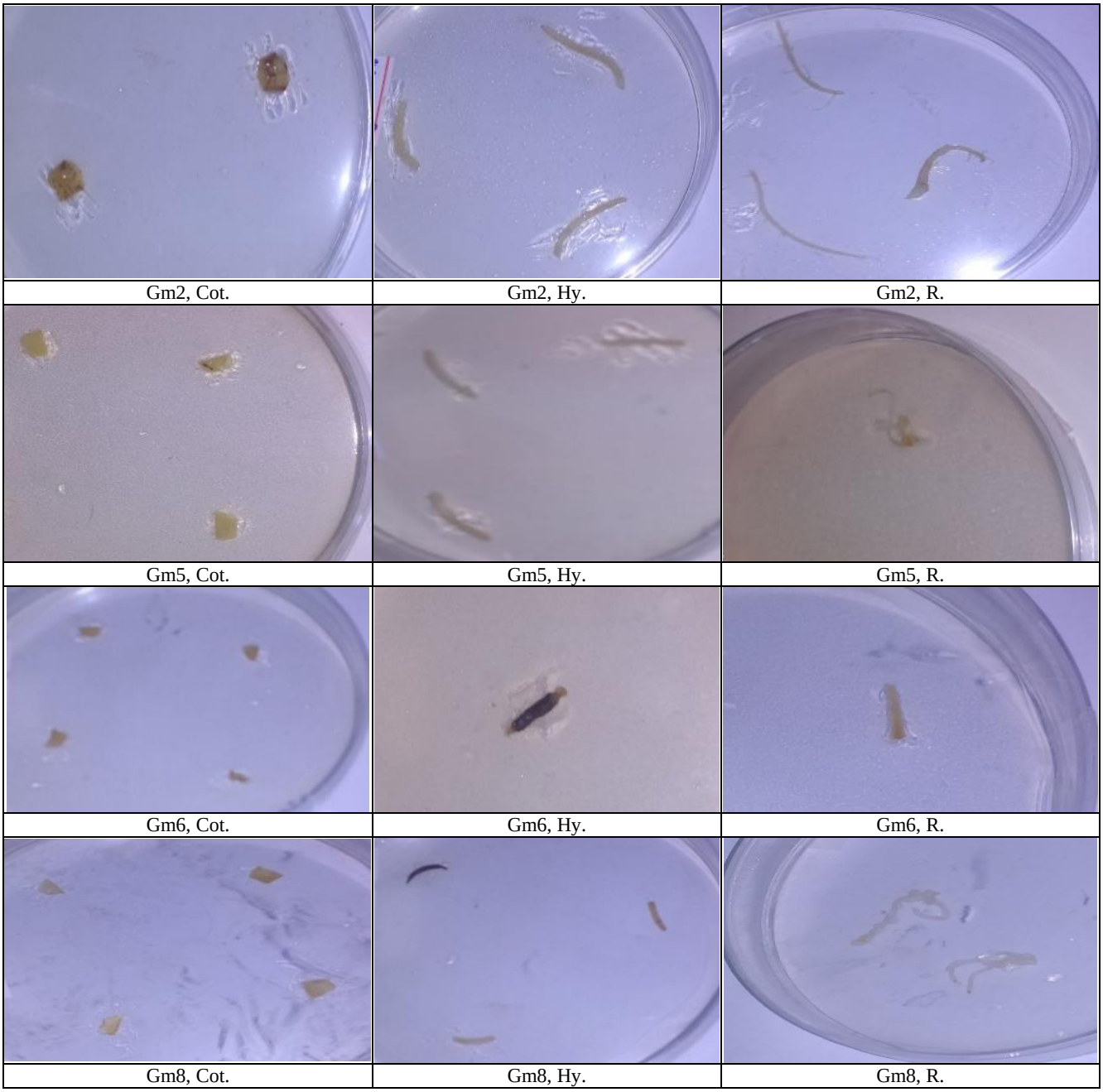
Fig 9: Callus initiation from different explants of *C. tetragonoloba* genotypes- 90 and 94 days after culturing on M3 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root



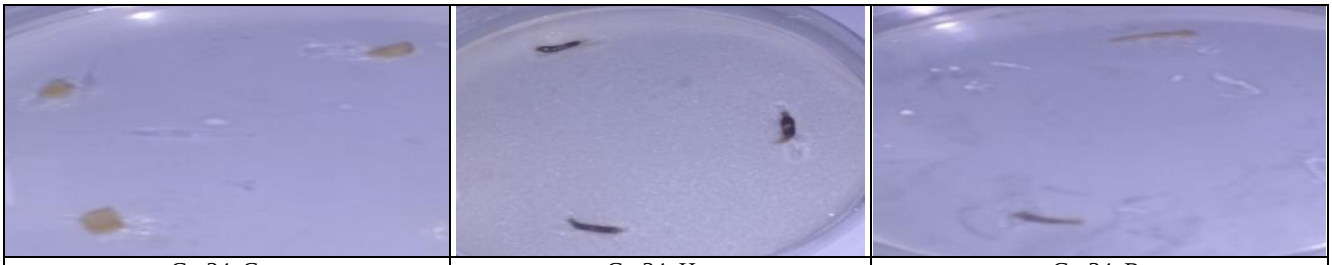


Gm34, Hy.	Gm34, R.

Fig 10: Callus initiation from different explants of *C. tetragonoloba* genotypes- 102 days after culturing on M3 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root

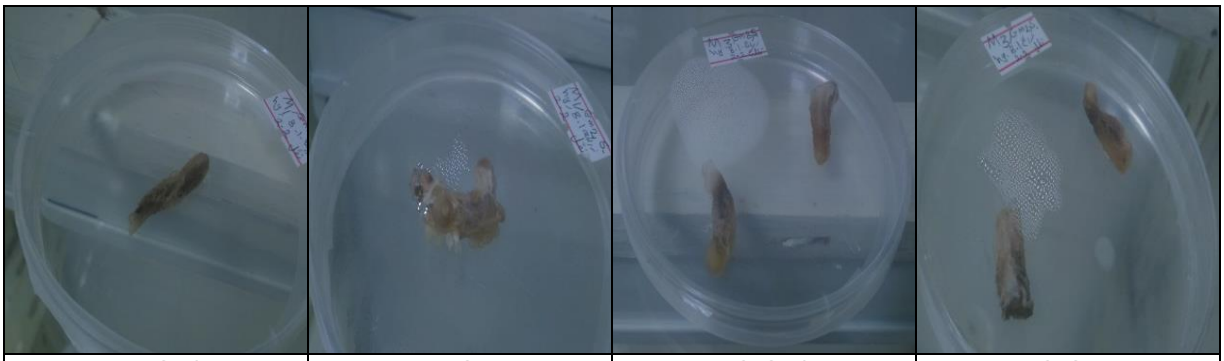


Gm2, Cot.	Gm2, Hy.	Gm2, R.
Gm5, Cot.	Gm5, Hy.	Gm5, R.
Gm6, Cot.	Gm6, Hy.	Gm6, R.
Gm8, Cot.	Gm8, Hy.	Gm8, R.



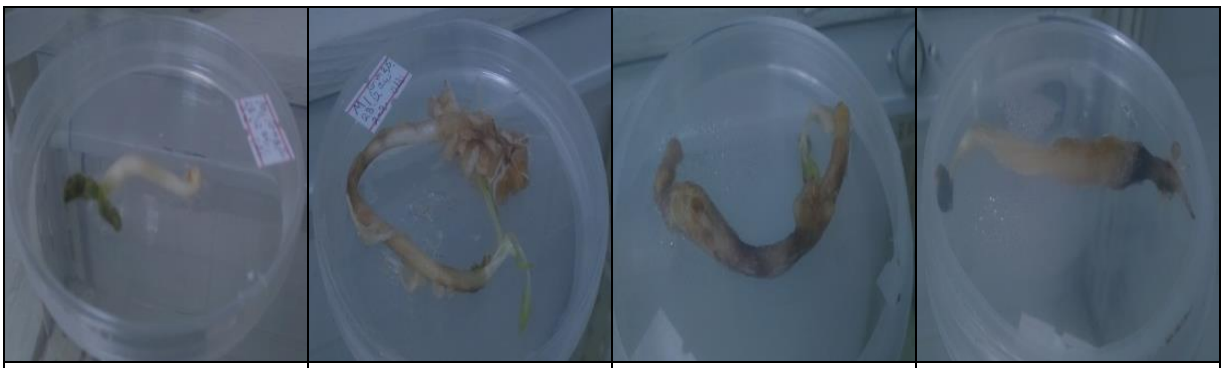
Gm34, Cot.	Gm34, Hy.	Gm34, R.

Fig 11: No callus formation in all genotypes from different explants of *C. tetragonoloba* genotypes on M2 medium, Cot.= cotyledonary leaves, Hy.= hypocotyl, R.= root



M1, Gm8	M1, Gm2	M3, Gm8	M3, Gm2

Fig 12: Callus initiation from hypocotyls of *C. tetragonoloba* two genotypes- 21 days after culturing on M1 and M3 media



M1,Gm8	M1, Gm2	M3, Gm8	M3, Gm2

Fig 13: Callus initiation from seeds of *C. tetragonoloba* two genotypes- 34 days after culturing on M1 and M3 media

4. Conclusion

It was concluded that when three explants of five genotypes of *C. tetragonoloba* were cultured on three types of media, results show that M2 (with NAA) failed to form callus for the five genotypes, while M1 (with IBA) and M3 (with 2-4, D) initiated callus for the five genotypes. M3 was better than M1 for callus formation for the *C. tetragonoloba* genotypes. M3 gave a good amount of callus tissues from the cotyledonary leaves explants of genotypes Gm2 and Gm8, and to the hypocotyl of genotype Gm5. Also, to the root explants of genotypes Gm5 and Gm34. M1 gave a good amount of callus tissues from the hypocotyl explants of genotypes Gm6, Gm8 and Gm34. No callus formation in hypocotyl of Gm8. M1 and M2 gave a percentage of 100% of initiation for all cotyledonary leaves explants of the five genotypes, also for the three explants of genotype Gm34. In M1 all initiation percentages were more than 50%, only root explants of genotypes Gm2, Gm6 and Gm8. Similar results were observed in M3, while hypocotyl and root explants of Gm2 were also less than 50%. The minimum time required for initiation was 6 days in the three explants of genotypes Gm6 and Gm34 in M1, and the last one was 30 days in the hypocotyl of Gm6 in M3. Organogenesis for the three explants show that M1 and M3 form a callus in the five genotypes of *C. tetrtagonoloba*, while M2 failed to form a callus for the five genotypes, M1 stimulated adventitious roots formation in hypocotyl explants of genotypes Gm6, Gm8 and Gm34. Initiated calluses were fibrous with creamy colour for the five genotypes. Callus initiatied in M3 medium from cotyle of *C. tetragonoloba* two genotypes of Gm2 and Gm8, while Gm2 formed callus in M1 medium better than Gm8 and the same effect was recorded for seedling cultures of the two genotypes.

5. Acknowledgement

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