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In Vitro Clonal Propagation and Phytochemical Analysis of *Momordica charantia* .Linn

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The explants from *in vitro* grown seedling were cultured on modified MS medium. Shoot, root and callus differentiation was obtained on MS medium supplemented with BAP, IBA and 2, 4-D respectively. Shoot as well as root differentiation was obtained on medium containing BAP+IBA/NAA. Multiple shoots with roots were obtained on MS medium without hormones(MSO).On Qualitative and quantitative estimation of flavonoids & steroids , presence of three flavonoids and three steroids was detected and the maximum amount was in 6-wk old callus cultures(2.90mg/g dw and 27.34mg/gdw respectively).while in different *in vitro* morphogenetic stages, the maximum amount of flavonoid and steroid content was in multiple shoots(2.96mg/gdw and 10.39mg/gdw).On supplementing the callus with different concentrations of cholesterol and salicylic acid significant recovery of diosgenin 6-wk old callus supplemented with 0.05mM concentration.

Keyword: M. charantia, Regeneration, Explant, Secondary metabolites

1. Introduction

Momordica charantia L. commonly known as bitter melon/gourd, a member of Cucarbitaceae, is a slender, tendril climbing, annual vine. Bitter melon is a common food item of the tropics and is used for the treatment of cancer, diabetes and many ailments (Cefalu *et al.*, 2008; Leung *et al.*, 2009; Modak *et al.*, 2007; Nahas *et al.*, 2009). It is a potent hypoglycemic agent (Basch et al, 2003; Singh et al, 2011) and its hypoglycemic actions for potential benefit in diabetes mellitus are possible due to at least three different groups of constituents in bitter melon. These include alkaloids, insulin like peptides, and a mixture of steroidal sapogenins known as charantin. Clinical studies with multiple controls have confirmed the benefit of bitter melon for diabetes (Raman & Lau, 1996). Alpha and beta momarcharin are two proteins found in bitter melon, which are known to inhibit the AIDS virus (Zhang, 1992). *M. charantia* plant has not been much investigated for its *in vitro* culture response. However, formation of callus is reported (Khanna & Mohan, 1973).

Plant tissue cultures have been investigated for industrial production of several useful secondary metabolites (Rao & Ravishankar, 2002; Wink et al, 2005). Among the natural phenolic compounds flavonoids are one of the wide spread groups also acting as chemotaxonomic markers (Harborne, 1985; Islam et al,2011).Various workers described that the phenolic substances are the active products of cellular metabolism and of great importance as they act as analog of growth hormones (Bray &Thrope, 1954; Subramanian & Nagrajan, 1969).

Phytosteroids are pharmacologically important for human life (Hardman, 1980). They are derivatives of cyclopentanoperhydrophenanthrene and include steroidal sapogenins, Steroidal glycosides, cardiac glycosides and hormones.

The steroidal sapogenin, diosgenin is found in many plants but it is obtained principally from *Dioscoria* roots (4 to 6% DW) for conversion to commercially useful drugs. Diosgenin is produced from different species of *Dioscoria* (Staba, 1985; Tomita & Uomori, 1974).

Table 1: Shoot production of M.charantia grown on various hormonal concentrations supplemented in MS medium and MS medium without hormones (MSO) : values are mean + SE (n=10 2)

Growth Hormones mg/l	**Shoot tip**		**Explant Nodal**		**Internodal**		**Shoot tip with node**	
BAP	Growth response	Number of shoots	Growth response	Number of shoots	Growth response	Number of shoots	Growth response	Number of shoots
0.5	Multiple shoots	4+0.54	Multiple shoots	4.7+0.58	Multiple shoots	4.9+0.61	Shoots with callus	4.5+0.57
2	Multiple shoots	4.1+0.53	Multiple shoots	4.3+0.53	Multiple shoots	4+0.54	Multiple shoots	4.2+0.51
4	Shoots with callus	1.5+0.25	Shoots with callus	1.6+0.24	Shoots with callus	1.7+0.23	Shoots with callus	1.5+0.25
6	Callus	-	-	-	Callus	-	Callus	1.8+0.25
Kn								
0.5	Multiple shoots	1.3+0.20	Multiple shoots	2.4+0.35	Multiple shoots	1.7+0.24	Shoots with callus	1.9+0.21
2	Multiple shoots	1.7+0.23	-	-	Multiple shoots	1.5+0.17	Shoots with callus	2.1+0.26
4	Multiple shoots	1.4+0.16	-	-			Shoots with callus	2.5+0.31
6	-	-	-	-			-	
MSO	Multiple Shoots with roots	3.2+0.36	Multiple Shoots with roots	4+0.24	Multiple Shoots with roots	1.5+0.25	Multiple Shoots with roots	4.5+0.57

Diosgenin is synthesized in all plant parts with higher concentrations in fruit of *M. charantia* (Basch, 2003; Taylor, 2004). Consequently the callus cultures of *in vitro* seedling are most appropriate *in vitro* system to produce them. The latter have several advantages, among them their growth rate and production patterns that are similar to plant in vivo (Flores, 1999).

Among the methods employed to enhance production of secondary metabolites is the use of Salicylic acid. Exposure to SA frequently induces the synthesis of secondary metabolites in plant (Benhamou, 1996; Namdeo, 2007; Jeong &Park, 2007).

One more successful venture in this regard is to supplement nutrient medium with one of the precursors of biosynthetic pathway so as to increase the biosynthetic potential of valuable products useful as pharmaceuticals and fine chemicals (Kim et al, 2004). Compounds added to media as precursors are to be transformed to economically important compounds (Thanh et al, 2005; Jeong &Park, 2007).

In present investigation an attempt was made for complete regeneration of *M.charantia* through multiple shoots, callus, and shoot apex, nodal and internodal explants. Besides this an attempt has been made to study the tissue cultures of this

plant for *in vitro* flavonoid and steroid production. Callus cultures of *M.* Charantia were exposed to SA and cholesterol in order to increase the productivity of diosgenin and /or its releases.

Table 2: The effect of growth hormones on average rooting of *in vitro* developed shoots of *M.charantia*

Hormones Mg/l	Shoot tip	Explant Nodal	Internodal
IAA			
0.5	R(+)	R(++)	R(+)
1	-	-	R(++)
2	R(+)	-	R(+)
3	R(++)	R(+)	-
4	R(+)	R(+)	-
5	R(+)	R(++)	-
IBA			
0.5	R(+)	R(+)	R(+)
1	-	R(++)	R(+)
2	R(+)	-	R(+)
3	R(++++)	R(+++)	R(++++)
4	R(++)	R(++)	R(++)
5	R(+)	-	R(+)
NAA			
0.5	R(+)	R(++)	R(+)
2	R(+)	R(+)	R(+)
3	R(+++)	R(++)	R(+)
4	R(+)	-	R(+)
5	-	R(+)	-

R = Rooting, +Poor,++ Moderate,+++ Good,++++ Very good

2. Materials and Methods

Seeds of bitter gourd (*Momordica charantia Linn. cv.* Faizabadi) were collected and surface sterilized. The seeds were then inoculated on 15 ml aliquots of 0.8% agar gelled MS Medium (murashige & Skoog, 1962) and incubated in dark for 20 days. When the radical emerged from the seeds, they were transferred to incubation at $25+2^0$C with a 16 h photoperiod provided by cool fluorescent light (50 µ $Em^{-2}s^{-1}$) (Phillips, India). After 5 weeks, from 15 cm long seedlings (Fig 1A), 5-6mm shoot tip explants, 20-25 mm long nodal and Internodal explants were dissected and inoculated on MS medium with various concentrations and combinations of growth regulators . The comparative morphogenetic responses of the explants taken from the *in vitro* grown seedling to plant hormones were observed regularly (Table1-2). Each treatment had 10 replicates of culture and experiments were repeated twice.

Five weeks after root initiation the plants were hardened in 5 X 5 cm portrays filled with cocopeat + Soil (1:1) and kept in a moist saturated greenhouse having 60-80 % humidity. The plants were irrigated twice daily. After three weeks the established plants were transplanted to 15 cm pots containing, soil + biofertilizer for further growth.

Extraction and analysis of flavonoids and steroids was carried out by using the methods of Ozeki & Komamine (1985) Wong and Francis (1968), Mabry et.al (1970) and Tomita et.al (1970) respectively.

Table 3: Flavonoid content in plant parts, callus cultures, *in vitro* morphogenetic stages.Values are mean+SE (n=10x2)

Flavonoid content(mg/gdw)				
	Luteolin	**Kampherol**	**Quercetin**	**Total**
Fruit	0.12+0.14	0.56+0.065	0.20+0.021	0.88+0.099
Seed	0.13+0.013	1.13+0.129	0.38+0.039	1.64+0.18
2week	0.09+0.0009	1.40+0.145	0.34+0.028	1.83+0.180
4week	0.14+0.013	1.68+0.159	0.46+0.039	2.28+0.220
6week	0.20+0.021	2.13+0.210	0.57+0.056	2.90+1.290
8week	0.13+0.012	1.48+0.145	0.35+0.036	1.96+0.194
***In vitro* Grown seedling**	1.40+0.140	0.09+0.009	0.34+0.034	1.83+0.182
callus	1.50+0.148	0.15+0.013	0.33+0.032	1.96+0.192
callus with rooting	2.13+0.210	0.20+0.018	0.57+0.056	2.90+0.290
regenerated shoots	1.70+0.160	0.14+0.014	0.46+0.045	2.30+0.230
multiple shoots	2.15+0.210	0.22+0.020	0.57+0.056	2.96+0.288

2.1 Elicitor and Elicitation Procedure

The liquid MS medium with NAA (2mg/l) +BAP (0.5mg/l) +2, 4-D (2mg/l) was prepared. Callus was transferred to each flask and liquid cultures were grown on reciprocal shakers (125rpm; 5cm/stroke) for 6 days, on the 6th day liquid MS media was supplied with three concentration (0.1mM, 0.05mM, 0.025mM) of salicylic acid and the cultures were again grown on same shakers for exactly 24h.The tissue grown on different media were harvested and weighted again and dried at room temperature. The callus samples were powdered and subjected to qualitative and quantitative estimation of diosgenin.

2.2 Feeding Precursors

An experiment was conducted in which three concentrations of cholesterol (0.1mM, 0.05 m M, and 0.025mM) were mixed with liquid MS media supplemented with NAA (2mg/l) +BAP (0.5mg/l)+2,4-D (2mg/l) separately. Later, callus was transferred to the liquid MS media having three concentrations of cholesterol. The MS media without cholesterol served as control. The liquid cultures were grown on shakers (125rpm; 5cm/strokes). The tissue grown in different media were harvested at regular time intervals of 2, 4, and 6 and 8 weeks after subculturing, dried and powdered.

Table 4: Steroid content in plant parts, callus cultures, *in vitro* morphogenetic stages of *M.charantia* L.Values are mean+SE(n=10X2)

	Steroid content(mg/gdw)			
	Stigmasterol	***B*-sitosterol**	**Diosgenin**	**Total**
Fruit	9.17+1.04	9.27+1.06	1.09+0.13	19.53+2.2
Seed	10.17+1.22	10.10+1.14	absent	20.81+2.35
Aerial parts	11.55+1.32	12.48+1.4	absent	23.79+2.6
2week	1.11+0.115	2.60+0.285	2.45+0.0281	6.16+0.068
4week	1.72+0.186	3.60+0.391	3.41+0.362	8.73+0.939
6week	2.5+0.283	3.72+0.416	3.22+0.365	9.45+1.064
8week	2.00+0.215	3.35+0.382	2.64+0.293	7.99+0.89
***In vitro* grown seedling**	1.11+0.130	2.60+0.288	2.45+0.275	6.16+0.70
Callus	1.90+0.196	3.25+0.370	2.45+0.276	6.60+0.73
Callus with rooting	2.51+0.284	3.72+0.422	3.22+0.364	9.45+1.09
Regenerated shoots	1.69+0.188	3.20+0.364	3.08+0.345	7.97+0.92
Multiple shoots	2.63+0.288	3.93+0.388	3.78+0.385	10.39+0.939

Table 5: Diosgenin in Callus Cultures of *M.charantia* with Various Concentration of Salicylic Acid.

Concentration of Salicylic acid	**Diosgenin (mg/gdw)**
Control	7.61+0.781
0.025mM	8.03+0.801
0.05mM	8.98+0.855
0.1mM	8.32+0.799

Values are mean +SE (n=10x2)

2.3 Chemical Analysis

To estimate diosgenin callus samples were soxhlet extracted in 30% hydrochloric acid (v/v) for 4h.Each sample was then dried at 60^0C for 8h and then soxhlet extracted with benzene for 24h, filtered, dried in vacuum, dissolved in chloroform, co-chromato graphed separately with standard steroids as markers on TLC plates. These plates were developed in a chromatographic chamber containing solvent mixture (benzene; ethyl acetate 85:15) (Heble et. al.1968). These plates were sprayed with 50% sulphuric acid and heated at 110^0 C for 10-15 minute.

2.4 Quantitative Analysis

For UV spectrophotometric analysis callus extracts were compared with standard diosgenin at absorption maxima of 450 nm. Standard curves for standard steroids were prepared for reference. Optical density values of tissue extracts were measured and compared with standard curve for quantification of diosgenin in the samples. Each treatment had10 replicates of culture and experiments were repeated twice.

Table 6: Diosgenin content in callus cultures of *M. charantia* L.

In weeks	Control	0.025mM	0.05mM	0.1mM
2	3.41+0.387	4.11+0.467	5.12+0.555	4.98+0.567
4	4.54+0.507	5.63+0.645	6.71+0.765	6.11+0.760
6	6.55+0.563	7.13+0.728	8.34+0.95	8.12+0.925
8	5.13+0.58	6.21+0.70	7.11+0.811	7.02+0.656

Values are mean+SE (n=10X2) Time Diosgenin Content mg/gdw

3. Results and Discussion

M. charantia a member of family cucarbitaceae is an important vegetable and medicinal herb. It functions as a hypoglycemic agent by regulating carbohydrate metabolism. Being a tropical crop, it does not find any organized cultivation in temperate areas.

Fig 1: A-Seedling; **B**-Seedling with Profuse Rooting **C**-Callusing on Shoot Explants; D-Rooting and Callus Formation on Shoots; **E**-H-Shooting from Callus; **I**-Rooting and Callusing on Shoot Explants.

M. charantia belongs to family Cucurbitaceae. *Cucumis melo* and *C. sativus* are two plants of this family which have attracted the attention of scientists for regeneration because they are some of the major vegetable crops of tropical countries. There are two ways to regenerate plants through the initiation of adventitious buds or through somatic embryos (Torrey, 1966).

Regeneration through organogenesis via shoot formation in *C. sativus* was reported on MS media + IAA +K and MS media +NAA+BAP with the best shoot growth obtained through a balance of both auxins and cytokinins (Kim et.al.1988). Regeneration from callus of *C. melo* was also reported (Moreno et.al., 1985).

Fig 2: A, B-Multiple Shoots; C, D-Multiple Shoots with Roots; E-Rooting On Multiple Shoots

In the present study, shoot and leaf explants were taken from aseptically grown seedling. tip, nodal and Internodal explants. Cytokinin concentration of 0.5 mg/l to 6 mg/l produced shoots after 20 days in culture and the best response was observed on media containing 0.5 mg/l and 2 mg/l BAP, while IBA/NAA were suitable for rooting with best response at 4 mg/l IBA and

2mg/l NAA, the root formation was observed after 22 days in culture. Callus was formed on 2,4-D, with profuse callusing at 2mg/l of 2,4-D.A combination of NAA+BAP+2,4-D was most effective for callus formation with best response in 2mg/ lNAA + 0.5 mg / l BAP + 2 mg/l+2,4-D (Fig 1B).

Regeneration of shoots from callus was on BAP (Fig1C) and regeneration of shoots as well as roots from callus formed on explants taken from *in vitro* grown seedling and shoot tip, nodal and Internodal explants taken from *in vitro* grown seedling was on NAA+BAP/IBA+BAP with best response at 2 mg/l NAA+0.5 mg/l BAP and 4 mg/l IBA + 2mg/l BAP (Fig1D). The shoots were 55 mm in length with 2-3 nodes and 1-2 fully expanded leaves, after 22 days. There was de novo regeneration of shoots from callus and different explants.

This is in agreement that (Cheng & Voqui, 1977; Murashige, 1961; Thrope & Patel, 1984) the tissue organ used as a source of explant can also be a determinant for the success of a plant tissue culture.

Fig 3: Rooting and Shoot Development from Shoot Explants

Our results support the observation (Kim et.al.1988) that organogenesis is determined by auxins and cytokinins. Regeneration from *C. melo* and *C. sativus was* reported, respectively through somatic embryogenesis as well as organogenesis. In the present study regeneration was observed through organogenesis only. This is in confirmation with the views (Matsuoka & Hinata, 1979; Rajasekaran et.al, 1983; Reynolds, 1986) that differences in the culture conditions required for organogenesis and somatic embryogenesis are not well defined because very few examples exist where both embryogenesis and organogenesis occur.

It was found that auxin free media containing kinetin stimulated the production of complete *C. sativus* plants with minimal callus (Handley & Chambliss, 1979). On low concentration of cytokinin initiation of multiple shoots from explants of *C. sativus* was observed with a dramatic increase in callus production and reduction in number of shoot produced after the addition of IAA (Rhonda &William, 1990).

In the present experiment three type of multiple shoot developments were observed on BAP, a low concentration of BAP (0.5mg/l) resulted in high shoot production with minimal callus (Fig 1E), on higher concentration of BAP shoot production was moderate and this was associated with increased callus growth (Fig 1G; Table 1, 2).Therefore MS medium without hormones was chosen as basal medium for further work. On this medium multiple shoots as well as roots formed (Table1; Fig1H). The shoots attained a length of about 60-mm with 2-3 nodes and 1-2 fully expanded leaves. While some callus was associated with these shoots it was only at the edge of explant and not on the site of *de novo* shoot production.

This is in agreement that (Handley & Chambliss, 1979; Rhonda & William, 1990) that multiple shoots can be formed on MS medium without hormones. The single factor determining organ initiation is the relative quantities of auxins and cytokinins (skoog & Miller, 1957). When tissues *in vitro* do not appear to require exogenous supply of auxins and cytokinin, it may be that

sufficient endogenous levels of hormones exist in the culture system for organogenesis.

The shoots regenerated from callus, seedling explants and from multiple shoots were separated and transferred on MS medium containing different concentrations of rooting hormones, best response was on 3mg/l IBA, and the roots were developed roots in ten days (Fig1I; Table2). The complete plantlets thus formed, were hardened in green house and transferred to pots where 40% plants survived successfully (Fig1J).

The secondary metabolites are of immense importance for use as commercially as well as biologically active compounds. Flavonoids are naturally occurring phenolic compounds, which have a widespread distribution in intact plants and have been found in a number of tissue cultures.

TLC analysis showed that three flavonoids were present in fruits, seeds, *in vitro* grown seedling, callus and different developmental stages; these were further identified with UV light in which three spots gave characteristic fluorescence at specific Rf values. These were comparable to their respective standard compounds (Kaempferol-Rf-0.86, Luteolin-Rf-0.56, quercetin-Rf-0.78).

Quantitative data revealed that the total flavonoid was more in 4 weeks old mature seeds (1.64mg/gdw) as compared to 2 weeks old mature fruits (0.88 mg /gdw). In seeds the total kaempferol content (1.13mg/gdw) was found to be higher (0.56mg/gdw) than fruits (Fig 4; Table3).

In callus cultures, the maximum amount of total flavonoid content was observed in six-week-old callus (2.90mg/gdw) and minimum was in 2 weeks old callus cultures (1.83mg/gdw). Individually in 6 week old callus cultures the total kaempferol was maximum (2.13mg/gdw) and luteiolin was minimum (0.20mg/gdw) (Fig4; Table3).

Some valuable biochemical are only synthesized or synthesized in greater quantity by differentiated tissue or are only accumulated in specialized organs or tissues. The cell differentiation follows specific biochemical and morphological principles. The formation of secondary plant products is integrated parts of a differentiation process. Synthesis and accumulation of secondary metabolites can be endogenously controlled, development dependent differentiation process and/or can be regulated by various exogenous factors.

Secondary plant product is an expression of a particular stare of cell differentiation, which in turn can be influenced by particular signals. In some plants initiation of morphological differentiation represents such triggering signal, different metabolite require degrees of tissue differentiation (Vinterhalter et al, 2008; Gao et al, 2004; Endress, 1994 ;). Therefore it is necessary to maintain differentiated and organized tissue for the purpose of extraction of the required drugs.

Among the different developmental stages maximum amount of total flavonoid content was in 6 weeks old multiple shoots (2.96mg/gdw), followed by 6 weeks old callus with rooting (2.90mg/gdw), 6 weeks old regenerated shoots (2.30mg/gdw), 6 weeks old callus (1.96mg/gdw), 25 days old *in vitro* grown seedling (1.83 mg /gdw) (Table 12). Individually the Luteolin was the highest in all the morphogenetic stages followed by Quercetin and Kampherol (Table 3). This is in agreement that some biochemical are only synthesized or synthesized in greater quantity by the differentiated tissue system.

Sterols are ubiquitous in higher plants and probably also in plant tissue cultures. *B*-sitosterol has been reported from *Nicotiana tabacum* [(Beneveniste *et al.*1966), *M.charantia* (Garver *et al.*1980; Khanna & Mohan, 1973) and stigma sterol is reported from *Dioscorea tokoro* [(Tomita *et al.*, 1970] and *M.charantia* [(Khanna &Mohan, 1973).

On qualitative analysis of fruits, seeds, *in vitro* grown seedling, callus and different developmental stages, three spots were visualized under UV light on TLC, which gave characteristic fluorescence at specific Rf values. These were comparable to their respective standard compounds (Diosgenin-green, Rf 0.50, sitosterol-pinkish grey, Rf-0.90, stigmasterol-greyish violet, Rf-0.83). The characteristic colours were also developed corresponding to the respective authentic samples, when TLC plates

were sprayed with anisaldehyde reagent (Diosgenin-grayish violet, *B*-sitosterol-pink, stigmasterol-greyish violet) and with 50% sulfuric acid (Diosgenin-green, *B*-sitosterol-pinkish gray, stigmasterol-greyish violet).

Quantitative data revealed that the total sterol (*B*-sitosterol and stigmasterol) was maximum in the 12 weeks old aerial tissue (23.79mg/gdw). Presence of diosgenin was observed in fruits only (1.09mg/gdw).Individually *B*-sitosterol and stigmasterol were highest in the aerial parts (12.48mg/gdw; 11.55 mg/gdw) (Table4).

In callus cultures, the maximum amount of total steroid content was observed in six-week-old callus (27.34mg/gdw) and minimum was in 2 weeks old callus cultures (12.12mg/gdw)(Table4).

On analyzing the different developmental stages maximum amount of total steroid content was in 6 weeks old multiple shoots (10.39mg/gdw), followed by 6 weeks old callus with rooting (9.45mg/gdw), 6 weeks old regenerated shoots (7.97mg/gdw), 6 weeks old callus (6.60mg/gdw), 25 days old *in vitro* grown seedling (6.16 mg /gdw) (Table 4). Individually the *B*-sitosterol was the highest in all the morphogenetic stages followed by diosgenin and stigmasterol (Fig8; Table15).

The steroidal sapogenin, diosgenin is found in many plants but it is obtained principally from *Dioscorea* roots (4 to 6%dw) for conversion to commercially useful drugs. Diosgenin is produced from different species of *Dioscorea,* [(Staba, 1977; Tomita *et al.*, 1970].

Elicitors strictly speaking are compounds of biological origin involved in plant microbe interaction. In the context of product accumulation by plant cells, elicitors are mediator compounds or stress agents. Production of secondary metabolite in tissue culture can be increased by use of elicitors [(Ebel, 1986).

There are some secondary metabolites which appear to be associated with the hypersensitive reactions which constitute early warning system, emanating signals to other cells /tissues in order to prepare them for secondary infection. In plant-pathogen interaction jasmonic acid and salicylic acid functions as endogenous signal compounds, thereby increasing the production of secondary metabolites by inducing the expression of defense related gene (Tatiana, 2000). There is no report on the effect of salicylic acid, when fed in the nutrient medium at very low concentration, on the production of diosgenin.

Production of secondary metabolites is usually most important in tissue that is farthest from meristmetic activity and in which biochemical maturation is advanced (Endress, 1994). Therefore, in the present study six-month-old callus formed on root and shoot explants of *in vitro* grown seedling was supplied with different concentrations of salicylic acid.

The presence of diosgenin was observed by thin layer chromatography coinciding with its standard sample in Rf values (Diosgenin-0.50). The color reaction test when sprayed with 50% H_2SO_4 fluorescent spot coinciding with their respective standard marker (Diosgenin-Green).

Quantification of diosgenin was achieved by using spectrophotometric analysis. Maximum amount (8.98mg/gdw) of diosgenin was from the callus sample having 0.05mM concentration of salicylic acid. In all three-callus samples, diosgenin was more in amount when compared to control (7.61mg/gdw) (Table5).

The accumulation of diosgenin in callus samples is a rapid response to the addition of salicylic acid, within 24 h after the addition of elicitor, diosgenin concentration reached to its highest level. Very low diosgenin was detected in control. The formation of diosgenin in callus supplied with salicylic acid was correlated with the induction of enzyme of biosynthetic pathway, resulting in accumulation of secondary products suggesting that salicylic acid plays a role in the defense mechanism of *M.charantia.*

The accumulation of diosgeninnin was very rapid high amounts were accumulated after 24h and there was no further increase in diosgenin. The oxidation and/or polymerization of salicylic acid into the culture medium could explain this feature.

Increase in diosgenin content is in support (Rokem *et al.*1984) with the studies that salicylic acid acts as an endogenous signal compound there by increases the production of secondary

metabolite. An increase in diosgenin in the present experiment is the first report where the salicylic acid has played a definite role to enhance the content.

Precursors or intermediate precursors, when added in culture medium they increase the synthesis of various phytochemicals *in vitro* (Sanchez *et al.*1972). Diosgenin is produced from different species of Dioscorea(Tomita *et al.*1970) yields less than 1% and from 1 to 2.5% dry weight if grown on media containing cholesterol and 2,4 –D (Kaul *et al.*,1969).*Dioscorea deltoidea* cells incorporate cholesterol leading to formation of diosgenin (Tomita & Uomori,1974).

In the present findings callus cultures of *M.charantia* were grown for different time period (2,4,6,8weeks) on MS medium supplemented with different concentration of cholesterol (0.025mM, 0.05mM, 0.01mM).

The interaction between concentration of cholesterol and different time period showed that the highest diosgenin recovery was in 6-week-old callus, which was grown on MS medium supplemented with 0.05mM concentration (8.34mg/gdw) which was at par with 0.1 m Concentration of cholesterol (8.12 mg/gdw)(Table 6).

Diosgenin recovery in the tissue was highly significant in comparison to control. This is due to incorporation of cholesterol, acting as a precursor in the biosynthesis of diosgenin.

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