BRIEF COMMUNICATION

Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor

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Abstract

Shoot buds were induced directly on either side of midrib from adaxial surface of immature leaf explants in *Stevia rebaudiana* Bertoni five weeks after culturing in Murashige and Skoog's nutrient medium supplemented with 8.88 μM of N^6-benzylaminopurine and kinetin ranging from 4.65 to 6.98 μM. Immature leaves of 0.6 to 1 cm were found to produce best response (93 %) with a highest number of 4.93 shoot buds per explant. For elongation of regenerated shoot buds, MS medium supplemented with 30 g dm^{-3} sucrose and indole-3-butyric acid (IBA) ranging from 4.92 to 7.38 μM were found most suitable. The medium was further modified to suit bioreactor cultivation of regenerated shoots wherein the use of two-fold MS salts and 60 g dm^{-3} sucrose resulted in a high biomass yield of 50.68 g dm^{-3} (m/v) accounting for about 590 micro-cuttings in three weeks. Best rooting of micro-cuttings occurred in half strength MS medium supplemented with IBA ranging from 4.92 to 7.38 μM, 15 g dm^{-3} sucrose and gelled with 0.8 % agar. Rooted plants were successfully established in substrate containing sand, *Vermicompost* and garden soil in equal proportions and grown in greenhouse. This is the first report on direct shoot regeneration from *Stevia* leaves.

Additional key words: auxin, cytokinin, *de novo* shoot regeneration, micro-cuttings, micropropagation, rooting.

Stevia rebaudiana Bertoni is a perennial herb belonging to the family *Asteraceae*, now being cultivated in many tropical and sub-tropical countries. The leaves of *S. rebaudiana* synthesize non-caloric thermo-stable intense sweeteners, mainly steviosides (the ent-kaurine glycosides) that find applications in food and pharmaceutical industries. Purified stevioside imparts about 300-times higher sweetness than sucrose (0.4 % solution) and hence needed in low volumes. Another glycoside, namely rebaudioside-A, which is about 400-times sweeter than sucrose is also present in *Stevia* leaves. Therefore, there has been a large interest in *Stevia* breeding and apart from conventional methods, tissue culture and genetic transformation protocols need to be developed.

In *Stevia*, plant regeneration has been achieved through organogenesis from different explants such as leaves (Sivaram and Mukundan 2003), axillary shoots (Bespalhok *et al.* 1992), stem tips (Tamura *et al.* 1984), suspension cultures (Ferreira and Handro 1988) and anthers (Flachsland *et al.* 1996). The reports on shoot regeneration from leaf explants were invariably through a callus phase. No reliable protocol for direct shoot regeneration from leaf explants of *Stevia* is available at present. Therefore, the objectives of the study were to induce direct shoot regeneration from leaf explants, confirmation of such regenerants through histological studies, scale up of so obtained shoots in a bioreactor and to establish the plants in field conditions.

The plants of *Stevia rebaudiana* Bertoni were procured from Tamil Nadu Agriculture University, Coimbatore, India and established in greenhouse. Shoot tips with a pair of fully opened leaves were disinfected by dipping in freshly prepared aqueous HgCl$_2$ (0.1 %, m/v) solution for 3 min followed by several rinses with sterile

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distilled water. These were transferred to MS medium (Murashige and Skoog 1962) with 30 g dm^{-3} of sucrose where the pH of the medium had been adjusted to 5.8 prior to adding agar (7.5 g dm^{-3}) for gelling. The cultures were incubated under 16-h photoperiod, irradiance of 40 µmol m^{-2} s^{-1}, and temperature of 25 ± 2 °C for 4 - 6 weeks. The leaves from aseptic shoot cultures were cultured on MS medium supplemented with growth regulators at various concentrations *viz.*, 0.57 - 11.42 µM indole-3-acetic acid (IAA), 0.49 - 9.84 µM indole-3-butyric acid (IBA), 0.45 - 9.00 µM 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.44 - 8.88 µM N^6-benzylamino-purine (BA) and 0.46 - 9.30 µM kinetin (Kn) where, MS medium without any growth regulator served as control. Observations on effects of growth regulators were recorded 7 weeks after inoculation.

The leaves from aseptic shoots grown on MS medium were used for *de novo* regeneration of shoots. The regeneration medium used was MS with various combinations of BA and Kn (Table 1), as these growth regulators supported good response towards shoot regeneration in previous experiment. These cultures were maintained for 7 weeks before recording the observations.

Once the medium with particular combination of growth regulators was selected for direct shoot bud regeneration from the leaf explant, the effect of the leaf length on the frequency of *de novo* shoot bud regeneration was evaluated. For this, the leaves from aseptic shoots maintained on MS with 30 g dm^{-3} sucrose at standard conditions mentioned above were used. The leaf explants ranging in length from < 0.5 - 2.0 cm were grown on MS medium supplemented with BA (8.88 µM) and Kn (4.65 µM) for 7 weeks and the data were recorded as percentage response as well as number of shoot buds regenerated per leaf explant.

The leaf explants showing protuberances, when observed under stereo-microscope (which generally occurred one week after incubation), were selected for histological study. Morphological changes on the leaf surface were routinely observed under stereo-microscope (*WILD M3Z*, *Leica*, Heerbrugg, Switzerland). The explants were then washed in sterile distilled water and placed between the slices of potato pith. Freehand sections of about 0.5 to 1 mm thickness were made, washed in distilled water and incubated in a staining solution containing safranin (1 mg cm^{-3}). The sections were mounted on a slide and observed under an inverted microscope (*Labovert*, *Leitz*, Wetzlar, Germany) and the responses were documented on photomicro-graphs.

The shoots emerging directly from the leaf explants which ranged in length from 0.3 - 0.5 cm were cultured on MS medium containing various levels of IAA and IBA and incubated for 3 weeks under standard conditions mentioned earlier and the observations were recorded in terms of total shoot length.

In order to develop liquid medium for the bioreactor production of shoots, the regenerated shoots were grown in Erlenmeyer's flasks containing 40 cm^3 of medium with various concentrations of MS salts (1, 1.5, 2 and 2.5 fold) and sucrose (15, 30, 60 and 90 g dm^{-3}) without adding any growth regulator. Approximately 300 mg of aseptic culture (3 pieces of stem cuttings with a single node each) was transferred to liquid medium and incubated for 3 weeks on a gyratory shaker set at 80 rpm. Here, the lateral buds grew further producing elongated shoots with 5 - 6 nodes each. Such nodes were aseptically trimmed and used to grow further as micro-cuttings in bioreactor.

The bubble column bioreactor used in the present study (*BIOFLO 111*, *New Brunswick Scientific*, Edison, USA) was made of a glass column (height 22 cm and diameter 14 cm) with a working volume of 1.75 dm^3. The upper lid of the bioreactor was provided with openings for air inlet, outlet, inoculation port and sampling port. Air was supplied through a glass sparger of 45 cm height and 7 cm diameter, molded into a circular shape at the bottom having pores of size 1 mm. An autoclavable basket made of wire mesh of 10.5 cm height and 8.5 cm diameter was used to provide anchorage for the culture. This anchorage had pores of 0.5 cm diameter at the bottom as well as sides and was placed at a height of 7 cm from the bottom of the bioreactor vessel supported by a stainless steel stand. The distance between sparger and the anchorage was kept at 4 cm (Thimmaraju *et al.* 2005). Liquid medium for shoot multiplication was double strength MS salts and 60 g dm^{-3} sucrose. About 10 g of aseptic shoots (100 pieces) with single node obtained from the shake flasks were inoculated into the bioreactor. The bioreactor was operated at an environ-mental conditions mentioned above and a continuous air flow at the rate of 33.4 cm^3 s^{-1} for 3 weeks.

After the complete run of 3 weeks, the reactor was dismantled and the shoots were aseptically removed and were cut in such a way that each cutting had at least two nodes. The cuttings were then transferred to rooting medium containing half strength MS salts, 15 g dm^{-3} sucrose and various concentrations of IAA and IBA. After three weeks, the rooted shoots were washed in running tap water and planted in polythene bags containing mixture of sand, *Vermicompost* and garden soil in equal proportions. These bags were incubated in a polyhouse where 100 % humidity was maintained. The humidity was gradually withdrawn after 10 d by which time the plants produced one pair of new leaves. After 2 - 3 weeks, the plants were transferred to garden soil mixture and maintained in greenhouse.

Each experiment was repeated twice with at least twenty leaf explants/shoots per treatment. All the observations and calculations were made separately for each set of explants and were expressed as mean ± standard deviation. The significance ($P < 0.05$) of the variables studied was assessed by a one-way analysis of variance (*ANOVA*) test using *Microsoft Excel Xp$^®$*. The mean separations were performed by Duncan's multiple range test for segregating means where the level of significance was set at $P \leq 0.05$ (Harter 1960).
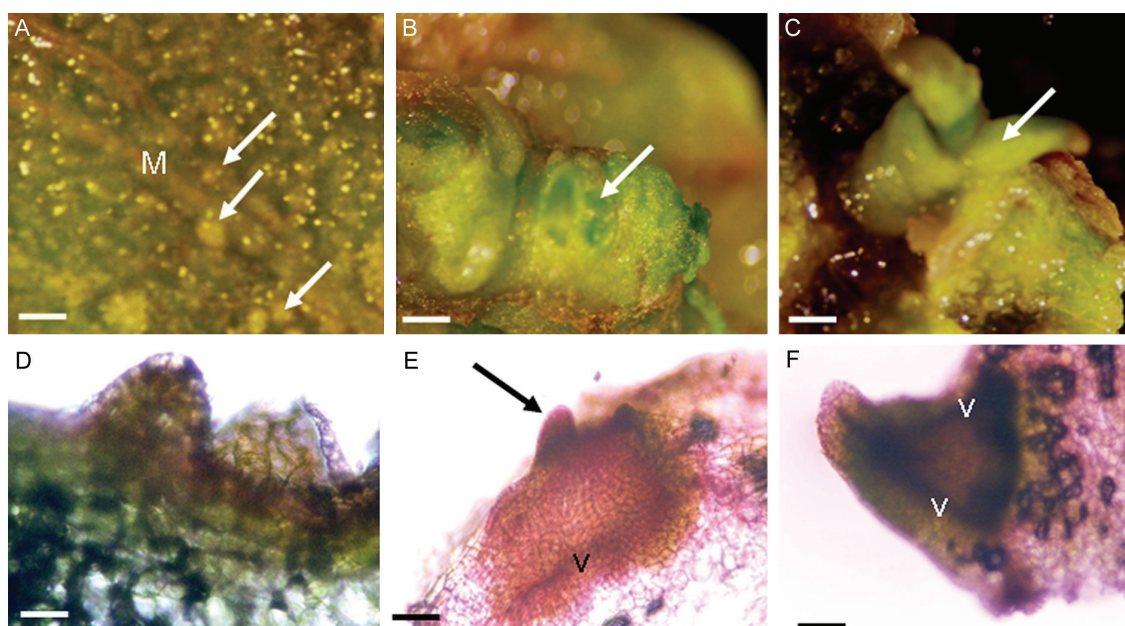
Fig. 1. *De novo* shoot regeneration from leaf explant of *Stevia rebaudiana* in MS medium supplemented with 30 g dm^{-3} sucrose, 8.88 µM BA and 4.65 µM Kn. *A* - Knob like shoot buds on either side of midrib 4 weeks after inoculation (M - midrib, *bar* = 2.5 mm). *B* - Further growth and greening of shoot bud 5 weeks after inoculation (*bar* = 2.5 mm). *C* - A pair of green leaves emerging from shoot bud (*bar* = 2.5 mm). *D* - Transverse section of leaf showing multiple shoot on leaf surface 4 weeks after inoculation (*bar* = 0.11 mm). *E* - Transverse section of leaf showing a group of cells organizing to form shoot primordia (V - vasculature, *bar* = 0.11 mm). *F* - Transverse section of leaf showing more organized shoot bud with its own vasculature 5 weeks after inoculation (V - vasculature, *bar* = 0.11 mm).
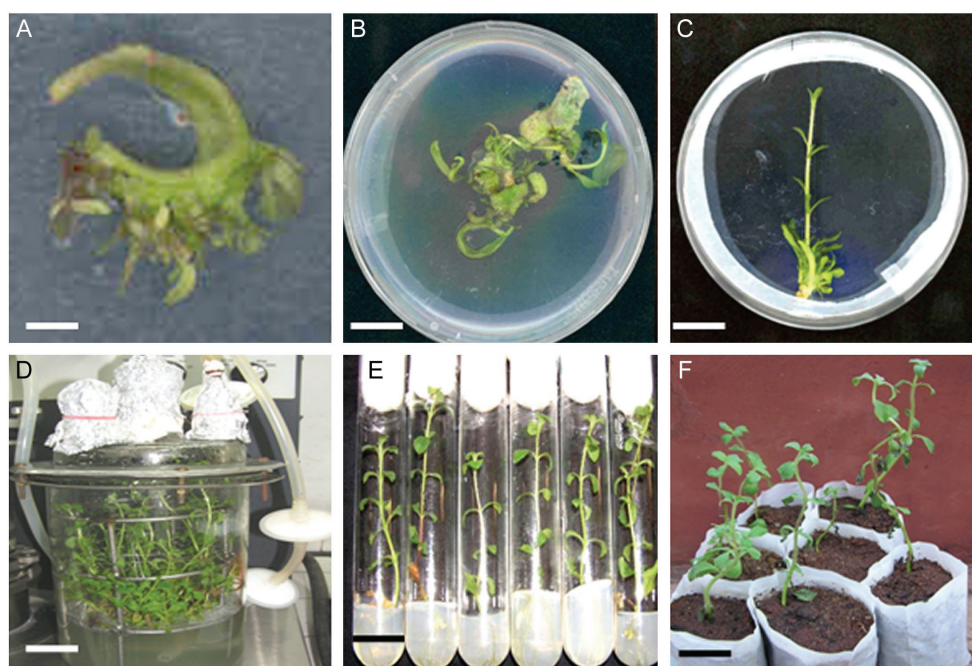

Fig. 2. *A* - Leaf surface showing shoot emergence 6 weeks after inoculation (*bar* = 0.25 cm). *B* - Rosettes of shoots arising from the leaf explant on MS medium supplemented with 30 g dm^{-3} sucrose, 8.88 µM BA and 4.65 µM Kn 7 weeks after inoculation (*bar* = 1.8 cm). *C* - Further elongation of regenerated shoots on MS medium supplemented with 30 g dm^{-3} sucrose and 4.92 µM IBA 3 weeks after inoculation (*bar* = 1.8 cm). *D* - Bubble column reactor jar showing mesh support holding shoots cultivated for 3 weeks in medium containing two folds of MS salts and 60 g dm^{-3} sucrose (*bar* = 4 cm). *E* - Rooting of bioreactor derived shoot cultures in half strength MS medium supplemented with 15 g dm^{-3} sucrose and 4.92 µM IBA 3 weeks after inoculation (*bar* = 2.5 cm). *F* - Regenerated plants established in garden soil mixture (*bar* = 4.6 cm).

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It is well established that a precise level of cytokinin would play a critical role in shoot organogenesis in various plants. Therefore in the present study, various combinations of growth regulators were tested where adventitious shoot buds could be successfully induced from the leaf surface in the presence of BA and Kn and not when other growth regulators were present. Further it was essential to fine-tune the level of BA and Kn required for achieving maximum number of shoot buds per explant. The leaf explants cultured on MS medium containing BA and Kn in various combinations appeared to enlarge in size by 3rd week and subsequently leading to the formation of adventitious buds on either side of the midrib by the end of 4th week. Initially, the shoot buds appeared as white knob like structures (Fig. 1*A*) which later turned green (Fig. 1*B*) leading to the formation of a pair of green leaves (Fig. 1*C*). In an earlier study, Kn in combination with NAA or IAA failed to induce shoot

Table 1. Combined effects of BA and Kn on leaf explants of *Stevia rebaudiana* responding towards *de novo* shoot regeneration (7 weeks after culture). Means ± SD of two separate experiments with 10 replicates each. Means within a column followed by same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

BA [μM]	Kn [μM]	Response [%]	Shoot number [explant^{-1}]	Shoot length [mm]
8.88	2.33	60.25	2.20 ± 0.86 [b]	5.73 ± 0.96 [a]
8.88	4.65	77.8	4.33 ± 0.45 [a]	3.73 ± 0.88 [b]
8.88	6.98	70.1	2.87 ± 0.91 [ab]	4.80 ± 0.94 [ab]
8.88	9.30	62.5	2.27 ± 0.96 [b]	3.47 ± 1.46 [bc]
6.66	9.30	65.98	2.43 ± 0.49 [b]	4.64 ± 0.98 [ab]
4.44	9.30	70.2	1.93 ± 0.88 [b]	3.22 ± 0.89 [bc]
2.22	9.30	75.24	0 [c]	0 [d]

formation on leaf explants of *Stevia* whereas BA was found to be more effective (Sivaram and Mukundan 2003). A combination of BA and Kn was found to be ideal for the *de novo* shoot regeneration in the present study. The media containing 8.88 μM BA and 4.65 or 6.98 μM Kn resulted in the formation of highest number

of shoots per explant which also showed uniform elongation at the end of 7 weeks (Table 1). However, the treatment with 8.88 μM BA and 2.33 μM Kn showed highest shoot elongation (Table 1).

The histological observations showed that the formation of multiple shoot buds (Fig. 1*D*) occurred *de novo* at the leaf surface where a group of cells appeared to organize and result in a shoot primordium (Fig. 1*E*). The response towards adventitious shoots mainly occurred around the midrib of the leaf explants, and rarely from regions of smaller veins but never from the lamina indicating that the presence of vascular cells appear to be crucial for the *de novo* regeneration of shoots (Fig. 1*A*). Kumar *et al.* (1998) also, made a similar observation in *Paulownia fortunei*. Contrarily, Tamura *et al.* (1984) showed the formation of shoots only from the margins of *Stevia* leaves in shoots cultured on a very high concentration of Kn (46.5 μM) for 40 d. This may probably be because of the accumulation of high amount of cytokinin that is known to induce adventitious shoot formation. Our observation of adventitious shoot emergence from either side of the midrib and especially more from the petiolar end indicate that there exists a physiological gradient in the explants from proximal to the distal end for *de novo* regeneration of shoot buds. Even in the case of laminar segment explants (with vasculature) regeneration was higher in those from petiolar end than those farther, as observed in apple leaf cultures (Welander and Maheswaran 1992). A series of experiments with *Cyclamen persicum* led to the conclusion that an increased density of vascular tissues towards petiolar end and hence, the level of phytohormones and other metabolites might be responsible for the increased shoot regeneration in this area (Karam and Al-Majathoup 2000).

It was noticed that the leaf length significantly affects shoot bud formation where, leaves of 0.6 to 1 cm showed best response (93 %) with a highest number of shoot buds per explant (4.93 ± 0.88). In many plant species, juvenile explants were found to be more regenerative than matured ones under *in vitro* conditions (Ibrahim and Pierre 2001, Wang *et al.* 2006). However the present study showed that leaf tissues at certain developmental stage were more regenerative than younger or older

Table 2. Influence of MS salts and sucrose on the growth performance of shoot cultures in shake flask and bioreactor. Means ± SD of two separate experiments with 10 replicates each. Means within a column followed by same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

	MS salts (fold)	Sucrose [g dm^{-3}]	Shoot length [cm]	Number of leaf pairs [shoot^{-1}]	Total fresh mass [g]	Biomass increase (fold)
Shake flask	1.0	15	4.83 ± 0.37 [bc]	3.1 ± 0.57 [c]	0.88 ± 0.08 [b]	2.93
Shake flask	1.5	30	6.01 ± 0.24 [b]	4.1 ± 0.88 [bc]	1.41 ± 0.02 [ab]	4.70
Shake flask	2.0	60	7.95 ± 0.34 [a]	7.2 ± 0.92 [a]	1.92 ± 0.03 [a]	6.40
Shake flask	2.5	90	7.66 ± 0.31 [a]	7.4 ± 0.88 [a]	1.83 ± 0.02 [a]	6.07
Bioreactor	2.0	60	13.32 ± 0.87	10.46 ± 1.25	88.70 ± 0.0	8.87

tissues. This response supports the view that the presence of specific gradient of nutrients as well as hormones in the explant interact with the externally supplied growth regulators resulting in specific metabolic pathways that promote organogenesis (Famiani *et al.* 1994).

The multiple shoot buds formed on shoot bud induction medium (combinations of BA and Kn) failed to elongate further on the same medium leading to the formation of either rosettes (Fig. 2*B*) or to necrosis. Therefore, the clumps of *de novo* regenerated shoots buds were transferred to media with various concentrations of IAA and IBA that are known to support elongation of shoots. Maximum elongation of the shoots was found on MS media supplemented with 4.92 (6.19 $\pm$ 0.63) or 7.38 (5.27 $\pm$ 0.34) μM IBA for 3 weeks (Fig. 2*C*), which also resulted in concomitant root formation.

The cultivation of shoots in bioreactor offers several advantages. Some of them being large number of plantlets can be easily produced, easy handling, stimulation of growth rate by forced aeration and suppression of apical dominance leading to stimulation of growth of numerous shoot buds into plantlets (Takayama and Akita 1994). Trials in this direction were done in the present study where an initial trial using liquid MS medium with 8.88 μM BA and 4.65 μM Kn resulted in bleaching and vitrification of shoots (data not shown). To overcome this problem, a separate experiment was conducted in shake flasks by varying the concentrations of MS salts and sucrose. Among the various media used for screening (Table 2), the medium containing two folds of MS salts and 60 g dm^{-3} sucrose was found ideal for the shoot culture in liquid medium resulting in the formation of robust dark green shoots and hence the medium was used for shoot multiplication in bioreactor. Development of multiple shoot clusters was clearly observed 10 d after inoculation. A nearly nine-fold increase in total fresh mass was reached within a period of 3 weeks (Table 2). The shoots were robust with good elongation and have 9 - 12 pairs of dark green leaves (Fig. 2*D*). At the end of

the culture period about 590 micro-cuttings (50.68 g dm^{-3}) could be obtained for transferring to rooting medium. In addition, fresh mass of the micro-cutting from the bioreactor was 140 mg in comparison with 100 mg of fresh mass on solid medium. Akita *et al.* (1994), while propagating *Stevia* shoots in a bioreactor, observed a higher increase in fresh mass of shoots than was obtained in the present study which could probably be due to the use of different source of shoot culture and a different type of bioreactor. More work on air mixing may probably lead to improved biomass yield that are being attempted in further work. It was also observed that most of the shoots propagated in the bioreactor had no roots. Hence, direct transfer of shoots from bioreactor to soil mixture for *in vivo* survival and acclimatization was not very successful leading to 50 % mortality. This indicated the need for development of a rooting medium.

The bioreactor grown shoots were removed, separated aseptically and were cultured on medium containing half strength MS salts, 15 g dm^{-3} sucrose and various levels of IAA and IBA. Among these combinations, media supplemented with 4.92 or 7.38 μM IBA were found to be the best, which resulted in the formation of 4 to 6 well-formed roots in almost all the shoots within 3 weeks (Fig. 2*E*). A similar result was obtained for solid medium derived shoots, where a slightly higher number of roots was observed (Sivaram and Mukundan 2003). However, the roots formed in the present study were good enough for their successful establishment in soil (Fig. 2*F*) with a survival rate of 94 %.

In conclusion, we report for the first time, a protocol to obtain direct shoot regeneration from leaf explants of *Stevia rebaudiana*. A medium composition was also developed for bioreactor cultivation of shoots that was followed by a rooting medium. Apart from this, the regeneration protocol developed in the present study appears highly useful for further work involving gene transfer and mutation breeding leading to genetic improvement of this crop.

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Dugan, F.M.: **The Identification of Fungi – An Illustrated Introduction with Keys, Glossary, and Guide to Literature.** - The American Phytopathological Society Press, St. Paul 2006. 176 pp. ISBN 0-89054-336-4.

The book is created as an introductory manual for identification of fungi specimens and cultures. On 176 pages it provides to user a huge number of clear diagrammatic drawings, which were synthesized and simplified from specimens and literature for representatives of principal families.

The manual starts with brief characterization of the major fungal groups: "Lower fungi", Zygomycota, Ascomycota, Basidiomycota, and Deuteromycetes, including typical life cycles for the phyla. As the book is not devoted to modern fungal systematics, classical non-phylogenetic classification is used, which corresponds with other current literature on fungi identification. Illustrated glossary greatly completes the manual introducing fundamentals of mycological terminology. The last part of the book contains references to publishers and distributors of mycological literature.

In conclusion, this book is certainly a useful tool for all working with fungal pathogens and I can recommend it to students or non-specialists as well as to scientists investigating plant diseases.

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