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# In Vitro Studies of Apple Varieties of Kashmir

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***Abstract:*** ***Apple is rosaceous pome fruit grown in temperate areas of the world for its high economic value. It is chiefly relished by people as fresh fruit but processed products like apple juice, canned apple sauce, apple jam and apple butter are also important table delicacies. Dehydrated apples, apple flour, apple dumpling, charoset (apple relish), apple haystacks are other important commercial products. The fruit contains appreciable quantity of sorbitol, sugars, organic acids and vitamins. It also has high medicinal value and serves as a stimulant for heart and as purgative, prevents constipation, reduces incidence of dental caries, helps to control obesity and supplies extra energy for heavy exercise. The pulp of apple fruit provides phytochemicals which serve as strong antioxidants and reduce the risk of some cancers, cardiovascular diseases, asthma, and diabetes. They also prevent oxidative stress and delay ageing. The other health benefits include better development of mental faculties. Apple trees when raised from seeds never produce quality fruits unless they are subjected to grafting. The traditional methods of propagation are time consuming, laborious and involve a lot of cost besides being skilful. Present investigation on in vitro studies of Ambri, Golden Delicious, Chambura and Maharaji apple cultivars has been taken in hand with an aim to develop complete micropropagation protocol for applying it in apple industry so as to boost the economy in the state. The maximum average shoot number produced per subculture of each shoot was found to be 34±0.82, 38±0.82, 42±0.71 and 48±0.82 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively the highest being in the Maharaji cultivar. The maximum average shoot number produced per subculture of each shoot was found to be 38 ± 0.81, 39 ± 0.80, 50 ± 1.35 and 52 ± 1.75 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively again highest recorded in the Maharaji cultivar and higher than the number obtained from the shoot apices of mature trees in all selected cultivars.***

***Keywords:-KN, TDZ, NAA, IAA, BA***

## I. INTRODUCTION

Apple is a rosaceous fruit tree, belonging to genus Malus. It is the most widely grown fruit tree in the world and is propagated in temperate regions of both northern and southern hemispheres of the world for its high economic value. The genus has five sections including 122 species and subspecies (Chadda and Awasti, 2004). Over 700 accessions introduced from different parts of the world have been tried and tested in India from 1950 (Surendra Gosh, 2006). Natural varieties of cultivated apple belong to Malus pumila Mill. While its hybrid varieties belong to Malus domestica Bork. Taxonomically apple is a dicotyledonous tree belonging to family Rosaceae with subfamily Maloideae (former Pomideae) having basic chromosome number 17. Cultivated apple has come into existence as a result of cross breeding among various species of Malus (Janick et al., 1996). Most cultivated varieties of apple are tetraploid but some are diploid (2n=34) and few are triploid (Mitra, 1996). Many Species called crab apples are cultivated as ornamental trees for their attractive flowers and fruits. Some notable examples are M. floribunda (Flowering Crab), M. baccata (Siberian Crab) and M. angustz'folia, (narrow leaved Crab Apple). Morphologically apple is a low spreading and round crowned hard wood tree having adventitious root, deliquescent stem, simple leaves, alternate phyllotaxy, pentamerous, actinomorphic flowers with creamy or pink petals and semi-inferior ovary. Fruit is pome having fleshy thalamus as its edible part.

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Its seed is nonendospermie and shows epigeal germination. From anatomical view point, apple is a hard wood tree having ring porous wood with vessels arranged in distinct concentric circles. Phloem parenchyma possesses crystals in different patterns. Leaves are dorsiventral having many layers of palisade tissue. Stomata are hypostomatic i.e., restricted to abaxial epidermis. Fruit has ten extra vascular strands. Embryological study reveals that flowers are generally produced on spurs of two or three year old shoots. Development of floral parts takes place in the order of calyx, androecium, gynoecium and corolla. Pollination involves allogamy as most commercial cultivars are self-incompatible. Pollen grains of many cultivars are also incompatible. For example, Golden Delicious is incompatible with Crispin. Pollination is entomophilous and mainly favoured by honey bees. Every commercial orchard possesses few recommended pollenizer trees. Fruit set is limited by endogenous ethylene formation. About 20% to 80% fruits are dropped off from an apple tree soon after fertilization, known as post blossom drop. Few apple cultivars like Cox"s Orange Pippin show parthinocarpy. Thining is one of the major techniques employed in apple to regulate fruit quality as too many fruits on the shoots tend to deteriorate quality of the fruit. Many chemicals like NAA, NAAM, nephthyl N-methylcarbamate (carbaryl) are commonly applied to induce flower thinning.From historic point of view, apple tree owes its origin in South eastern Europe and Tien Shan mountains of Kazakistan in Asia (Gastier, 2000) where vast forests of wild apple trees exist even today. The wild apple of ancient Asia, Malus pumila var. mitris, produced hundreds of tiny fruits that were sour having numerous, small, dark brown seeds, hardly a fruit that anyone would anticipate eating. It would never have made itself available to the modern table in its uncultivated form and it is only due to human efforts that we find it on table as principal fruit. The wild apple of Europe, the main ancestor of the domestic apple, is classified as Malus sylvestris. From economic view point, apple is the most important fruit grown all over the world. Nearly half of the production is consumed as fresh fruit and most of the remainder is processed into apple juice, canned apple sauce, apple jam and apple butter. Dehydrated apples, apple flour, apple, apple dumpling, charoset (apple relish), apple haystacks are other important commercial products. The fruit contains appreciable quantity sorbitol. and sugars (sucrose, glucose and fructose), organic acids (mainly malic and caproic acids) and Vitamins. The percentage composition of various ingredients from apple fruit is listed in Table 1.

| Nutritional Facts | | |
|---|---|---|
| Serving Size | One medium apple with skin (100g) | |
| Total energy per serving | 220 K Joules/50kcalories | |
| Constituent | Quantity | Percentage* |
| Water | 80-90 ml | 5.0% |
| Carbohydrates / Sugars | 10.39g | 2.6% |
| Dietary Fibers | 2.4g | 2.4% |
| Fat | 0.17g | 0.2% |
| Cholesterol | 0.0mg | 0.0% |
| Protein | 0.26g | 0.32% |
| Vitamin A | 3.0µg | 0.1% |
| Vitamin C | 4.6g | 8.0% |
| Vitamin $B_1$ Thiamine | 0.017mg | 1.0% |
| Vitamin $B_3$ Niacin | 0.091mg | 1.0% |
| Vitamin $B_5$ Pantothenic Acid | 0.061mg | 1.0% |
| Vitamin $B_6$ Pyrodoxin | 0.041mg | 1.0% |

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| | | |
|---|---|---|
| Vitamin $B_9$ Folic Acid | 3.0µg | 1.0% |
| Potassium | 107mg | 2.0% |
| Calcium | 6.0mg | 1.0% |
| Magnesium | 5.0mg | 1.0% |
| Iron | 320mg | 1.0% |
| Zinc | 0.04mg | 0.1% |
| Phosphorus | 11.0mg | 2.0% |

Source: USDA nutrient database (2009)

*Percentages are relative to US recommendations (2009) From medicinal view point, apple murraba, widely used in India is regarded as a stimulant for heart. Fresh apple acts as purgative, prevents constipation, reduces incidence of dental caries, helps to control obesity and supplies extra energy for heavy exercise (M itra, 2004). The old saying “An apple a day keeps the doctor away” has been reconfirmed as the pulp of apple fruit has been found to be the second richest source of phytochemicals like quercetin, catechin, phloridzin and chlorogenic acid, all of which are very strong antioxidants and reduce the risk of some cancers, cardiovascular diseases, asthma, and diabetes. They inhibit cancer cell proliferation, decrease lipid oxidation, and lower cholesterol. Thus they prevent oxidative stress and delay ageing. The other health benefits include better development of mental faculties. The phytochemical composition, however, varies greatly in different varieties of apples, and there are also small changes in phytochemicals during the maturation and ripening of the fruit. Storage has little to no effect on apple phytochemicals, but processing greatly affects apple phytochemicals (Boyer and Liu, 2004). Apples are sub-acid foods that have a rich pectin content and have proven rather beneficial to diabetes patients. Pectin, one of the most potent components in apples, acts as a detoxifier of the body by supplying an inordinate amount of galacrturonic acid. This helps to remove harmful waste from the bloodstream and can lower a diabetic"s insulin requirements by up to or even more than 35%. Apples are also helpful for both depression and weight loss and are considered a negative calorie food, as it takes more calories to digest them than they provide to the body. They release certain chemicals into the body which helps in the synthesis of glutei acid, and controls the destruction of various nerve cells (Boyer and Liu, 2004). Apples appear in many religious traditions, often as a mystical or forbidden fruit. One of the problems of identifying apples in religion, mythology and folktales is that as late as the 17th century, the word "apple“ was used as a generic term for all (foreign) fruits other than berries, but including nuts. This term may even have extended to plant galls, as they were thought to be of plant origin (oak apple). When tomatoes were introduced into Europe, they were called "love apples". In one old English work cucumbers have been called eorbaeppla (lit. "earth-apples'). Similarly in French and Dutch, the words for potatoes mean "earth-apples" in English. In some languages, oranges are called "golden apples" or "Chinese apples". Datura is called 'thornapple". In Latin, the words for 'apple' and for 'evil' are similar in the singular (Malus apple, malum evil) and identical in the plural (mala). This may also have influenced the apple's becoming interpreted as the biblical 'forbidden fruit'. The word Malus for apple comes from the Hittite mahla meaning "grapevine, branch" and has nothing to do with malum. The larynx in the human throat has been called Adam‘s apple because of the folk tale that the bulge was caused by the forbidden fruit sticking in the throat of Adam. The apple as symbol of sexual seduction has sometimes been used to imply sexuality between men and women. When held in Adam's hand, the apple symbolizes sin. But, when Christ is portrayed holding an apple, he represents the Second Adam who brings life. This difference reflects the evolution of the symbol in Christianity. In the story of Adam and Eve, the apple became a symbol for knowledge, immortality, temptation, the fall of man into sin, and sin itself. According to a popular legend, upon witnessing an apple fall from its tree, Isaac Newton was inspired to conclude that a similar 'universal gravitation' attracted the moon toward the Earth. Apple is the premier table fruit of the world and has been under cultivation since earliest times. Apple

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growing regions occur throughout the temperate zones of the world. In India, the major apple producing regions include Kashmir, Himachal Predesh, Utter Predesh, Kumaon, Assam and Nilgiri Hills. Kashmir is the leading apple producing state in India with annual production 60% of the total production in India. About 330 varieties of apple are known to have been under cultivation in Kashmir valley around 1972 but only a dozen are propagated at present on commercial scale. J & K state has remained popular for its indigenous apple variety Ambri from remote past. This variety has been utilised in breeding programme extensively, as a result of which a few hybrids namely, Lal Ambri (Red Delicious X Ambri), Sunehari (Ambri X Golden Delicious) and ASP-49 (Red Delicious with Ambri) were released. The new variety, ASP-49 is scab resistant, has more juice content, is sweet, and attractive in shape (Anonymous, 2009). Entire package of practices for apple has been developed and apple marketing has been well organised through establishment of HP Horticulture Processing and Marketing Corporation Limited (HPMC). Apple is now available throughout the country and RTS apple juice has become a popular drink. At present about 198000 hectares of land are involved in apple production and the total annual production from 2001 is depicted in Table 2.

Table 2: Total annual apple production in J&K state from 2001 -2010

| Year | Production in million metric tonnes |
|---|---|
| 2001-2002 | 0.909 |
| 2002-2003 | 0.953 |
| 2003-2004 | 0.956 |
| 2004-2005 | 0.953 |
| 2005-2006 | 1.151 |
| 2006-2007 | 1.222 |
| 2007-2008 | 1.311 |
| 2008-2009 | 1.332 |
| 2009-2010 | 1.372 |
| 2010-2011 | 1.852 |

(Source: Directorate of Horticulture, Kashmir, 2010)

All the commercial cultivars of apple are derived from carefully planned crossing and selection programme with specific objectives. New cultivars are more resistant to diseases and more productive than established cultivars (Alston and Watkins, 1983). M9 root stock shows high mineral uptake and induces early flowering. A new and distinct variety of apple tree rootstock Malus domestica x Malus robusta hybrid, G.935"has been developed in the current year in US (Telias, et al., 2009). The new patented variety is a dwarfmg rootstock, resistant to fire blight (Erwinia amylovora), crown rot (Phytophthora cactorum). Being efficient, precocious and highly productive, it can be propagated clonally and used as a rootstock as well as interstem of apple trees.

Traditionally apple trees for commercial orchards, in the valley, are propagated by grafting selected scion cultivars onto seedling or clonal root stocks or by budding. Seedling root stocks, however, have disadvantages of genetic variation which often lead to variability in growth and performance of the scion of the grafted plant. Clonal root stocks like Malling 28 are preferred for uniformity, maintenance of special characteristics and for specific influences they have on scion cultivars like disease resistance, growth and flowering habit (Gosh, 2004). These traditional methods of propagation are time consuming, laborious and involve a lot of cost besides being skilful. In View of these problems, use of newer techniques for producing large number of plants in less time, are preferred. Tissue culture techniques have been employed to explore possibilities for the propagation of apple through in

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vitro means from mid 20th century. Although the work was initiated by Letham (1958) and Saad and Bonne (1964), successful results in differentiating complete plants were obtained first by Jones (1967). According to him 60,000 green shoots can be produced by the techniques from single cultured shoot tip segment in 8 months. The method is thus less expensive and more efficient and hence a hit over the conventional methods of propagation. Tissue culture seems to be a logical method for the production of self rooted clonal apple trees as it has the potential to provide large number of plants in less time. Besides, it is expected to provide base for genetic improvement of the tree in terms of nutritional value and size of its fruit, disease resistance and stress tolerance. In our J&K state, this method is at pioneer stage and present work on four cultivars Ambri (pure and not hybrid one), Golden Delicious, Chambura and Maharaji (a continuation of M. Phil. work), has been taken in hand with the aim to develop complete protocol for its mass multiplication so that it could help in boosting the economy of the state in general and apple industry in particular. The three cultivars taken up for the study namely Ambri, Chambura and Maharaji are indigenous to Kashmir whereas Golden Delicious has originated in West Virginia, United States in 1910 through hybridization between Grimes Golden and Golden Reinette. Ambri cultivar having thick fruit skin, and longer shelf life, had been grown here long before Western introductions and continues to keep its superiority by Virtue of its crisp, sweet flesh and excellent aroma and represents an excellent dessert variety. Its fruits are medium sized oblong to conical in shape and blushed red in colour. Their ripening period is late October to November and thus they are seen in market in the month of November to March when most other fruits disappear. The fruits of Chambura cultivar are medium to large in size, yellow in colour with scattered light red patches, have flat base from pedicel side slightly tapering towards corolla and have highly juicy but slightly acidic flesh. They ripe in September to October and thus remain available in market in October and November. Maharaji apples are large in size, sour in taste, soft and bright red in color with conspicuous dots or streaks. They ripen in late October and are marketed in the month of November. Golden Delicious apples are medium to large in size, golden yellow in colour, sweet, juicy and crisp. Zimmerman (1984) developed explants and discussed various advantages of the technique over traditional cultivation practices. Achievements made on in vitro apple culture from 1958 to 2010 are quite encouraging.

## II. MATERIALS AND METHODS

The present research work on four cultivars of apple i.e. Ambri, Golden Delicious, Chambura, and Maharaji represents a continuation of M. Phil work and has been carried out with the aim assessing morphogenetic and organogenetic potential of different explants from mature trees and in vitro raised seedlings to develop complete protocol for mass propagation of the above mentioned cultivars.

Collection of Explants:-Different explants from four apple cultivars under reference (Ambri, Golden Delicious, Chambura, and Maharaji) were used as experimental material. The source material was obtained from mature trees and in vitro born seedlings. Seeds for the purpose were collected from freshly harvested apples obtained from different places in the Valley from time to time but bulk of the collection was obtained from different apple orchards at Zakura, Srinagar (4kms. from the University Campus). The explants used from mature trees were also obtained from different orchards at Zakura except Ambri cultivar which was obtained from Shopian and Pattan area in District Baramulla. The explants used as experimental material were 2-3cm long sprouts, apical buds, dormant buds, seeds (from mature fruits) and young leaves which were collected from mature trees of selected cultivars. The explants were cut by sterile razors and collected in polythene bags containing moist cotton to prevent wilting and were taken to laboratory. Explants were either processed for inoculations immediately or placed in a refrigerator overnight. In vitro born seedlings / shoots obtained after aseptic germination of mature apple seeds were also used as explants for raising cultures in all the cultivars.

Sterilization of Plant Material:-

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(a) Shoot Apices:-Shoot apices (0.5 cm long), obtained from young and actively growing shoots of 40-50 year old mature trees of the selected cultivars of apple were placed in enamel trays containing tap water with two to three drops of detergent (Labolene 5%) and a drop Tween-2O (surfactant). The explants were stirred gently and then washed with running tap water until all the traces of soap were completely removed. The explants were then placed in different sterilants for different time durations to attain complete asepsis and then rinsed 3 times with filtered water (obtained from water purifier) and finally with double distilled water. They were then kept in 10-25pM kinetin solution overnight (24hours) in refrigerator at 4°C to reduce leaching of phenolic compounds. Next day further processes were carried under laminar air flow cabinet. The kinetin solution containing phenolic exudates was drained out and the pre-washed explants were then again surface sterilized. A number of chemical sterilants were tried separately or in combinations to see their effect on percent contamination and survival after culture. The exposed cut end was trimmed off to eliminate toxic effect of sterilant which may have moved into cells during sterilization.

(b) Axillary buds:-Axillary buds of all the selected apple cultivars were obtained from the trees in three ways. During initials trails active axillary buds were directly collected from young twigs of mature trees, disinfected in the laboratory in the same Way as shoot apices and then inoculated. In middle trials dormant buds were collected from the field, disinfected chilled and then inoculated. In the following trials twigs possessing dormant buds were collected from the field, washed thoroughly and kept in vertical position in beakers with their cut ends dipped in kinetin(15-20pM) solution for few days until the dormant buds were forced to sprout. The sprouts were then disinfected with help of different sterilants for different time durations and then inoculated. Among the three procedures forced bud sprouts showed better results. The same procedure was followed for further axillary bud cultures.

(c) Nodal Stem Segments:-Like shoot apices, axillary (dormant and active) buds and nodal stem segments obtained from young and actively growing shoots of 40-50 year old mature trees of the selected cultivars of apple were subjected to same sterilization process alter trimming them to a length of 1cm.

(d) Leaf Segments:-Small, young and fresh leaves with intact petioles of all the four cultivars collected from juvenile branches of mature trees in the early morning were first brushed in petriplates and then sterilized with different sterilants for different time durations to standardise the procedure. The sterilized leaves were then cut into small circular discs or segments before inoculation.

(e) Seeds:-Seeds excised from mature fruits of all the selected cultivars were first washed under tap water using two to three drops of detergent (Labolene 5%) and a drop Tween-20 (surfactant), sterilized with different sterilants for different time durations, chilled at 4°C in GA3 (ZOμM) for 7-14 days and then washed (three times) with double distilled water. Then they were taken to laminar air flow cabinet where testa was removed. Testa free seeds were re-sterilized with different sterilants before inoculation.

Selection of Nutrient Media:-Four nutrient formulations namely White's (1943) medium, Murashige and Skoog's (MS) (1962) medium, Quoirin's medium (1972) and Woody Plant.

Table -4-Composition of different media used for apple tissue culture

| S.No. | Medium | WM | MSM | QM | WPM |
|---|---|---|---|---|---|
| | | White (1943) | Murashinge and Skoog (1962) | Quoirin (1972) | Lloyd and Mc. Crown (1980) |
| | Concentration→ | Mg/L | Mg/L | mM | Mg/L |
| | Ingredients↓ | | | | |
| A | Macronutrients | | | | |
| 01 | $NH_4NO_3$ | - | 1650 | 5.0 | 400 |
| 02 | $KNO_3$ | 80 | 1900 | 17.8 | - |

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| | | | | | |
|---|---|---|---|---|---|
| 03 | $CaCl_2.2H_2O$ | - | 440 | - | 96 |
| 04 | $MgSO_4.7H_2O$ | 750 | 370 | 1.5 | 370 |
| 05 | $K_2SO_4$ | - | - | - | 990 |
| 06 | $KH_2PO_4$ | - | 170 | 2.0 | 170 |
| 07 | $(NH_4)_2SO_4$ | - | - | - | 556 |
| 08 | $Ca(NO_3)_2.4H_2O$ | 300 | - | 5.1 | - |
| 09 | $Na_2SO_4$ | 200 | - | - | - |
| 10 | $NaH_2PO_4.H_2O$ | 19 | - | - | - |
| B | Micronutrients | | | | |
| 01 | KI | 0.75 | 0.83 | 0.1 | - |
| 02 | KCl | 65 | - | | |
| 03 | $H_3BO_3$ | 1.5 | 6.2 | 0.1 | 6.2 |
| 04 | $MnSO_4.H_2O$ | 5.0 | 22.3 | 0.1 | 22.3 |
| 05 | $MnSO_4.H_2O$ | - | - | - | 0.1 |
| 06 | $ZnSO_4.7H_2O$ | 3.0 | 8.6 | 0.03 | 8.6 |
| 07 | $CuSO_4.5H_2O$ | 0.01 | 0.025 | 0.0001 | 0.25 |
| 08 | $Fe_2(SO_4)_3$ | 2.5 | - | - | - |
| 09 | $MoO_3$ | 0.001 | - | - | - |
| 10 | $CoCl_2.6H_2O$ | - | 0.025 | 0.0001 | - |
| 11 | $Na_2MoO_4.2H_2O$ | - | 0.25 | 0.001 | 0.25 |
| 12 | $Fe_2(SO_4)_3.7H_2O$ | - | 27.8 | 0.1 | 27.8 |
| 13 | $Na_2EDTA.2H_2O$ | - | 37.3 | 0.1 | 37.3 |
| C | Organics | | | | |
| 01 | Thiamine HCl | 0.01 | 0.1 | 0.0012 | 0.25 |
| 02 | Nicotine acid | 0.01 | 0.5 | 0.0041 | - |
| 03 | Pyridoxin HCl | 0.01 | 0.5 | 0.0024 | - |
| 04 | Glucine | 3.0 | 2.0 | - | 0.5 |
| 05 | Myo-inositol | - | 100 | 0.56 | 100 |
| 06 | Sucrose | 2.0% | 3.0% | 2.0% | 2.0% |
| 07 | Agar | 0.8% | 0.8% | 0.8% | 0.8% |

Medium (WPM Lloyd and Mc. Crown, 1980) were tried (Table 04) but all explants responded well on MS medium. Thus all trials were later on canied on MS (1962) medium. However, use of full strength salt formulations with media supplements yielded poor results to the reduced salt strength i.e. half salt strength. The medium was supplemented with different auxins ( 2,4-D, IAA, NAA and IBA), cytokinins (BA and Kn), gibberellin (GA3) and PG and TDZ in different concentrations and combinations and encouraging results were yielded.

Preparation of Stock Solutions:-Weighing of all constituents of a nutrient medium individually and their mixing was made to the highest level of accuracy. Concentrated stock solutions of major salts, minor salts, myoinositol, Iron source, vitamins and phytohormones were prepared on need basis which not only saved time but was more accurate. The stock solutions were kept in

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dark glass bottles and stored in refrigerators. The strength and composition of stock solutions is depicted in Table 5. Stock solutions of phytohormones were prepared by dissolving 10mg of each hormone in 100ml of double distilled water (DDW). Initially acidic hormones (auxins and Gibberellins) were dissolved in little quantity of NaOH (0.1%) while basic hormones (Cytokinins) were dissolved in HCl (0.1%) and then DDW was added to make final volume. All stock solutions were stored at 4°C in refrigerators. Readymade nutrient media were also used.

Preparation of Nutrient Medium:-For all trials the medium was prepared in sterile vials of Borosil glass. Required quantities from (pre-prepared) stock solutions of MS major and minor salts, vitamins and myo-inositol were mixed together for one litre basal medium. This was followed by the addition of phytohormones from their stock solutions as per the need (Table 5). Double distilled water was added to increase the volume of the solution. Sucrose (300) was added and allowed to get dissolved properly. pH of the solution was adjusted between 5.2-5.8 by adding NaOH (0.1N) or HCl (0.1N) drop by drop. Final volume of the medium was adjusted, by adding more double distilled water, before the medium was gelled with 0.8 % agar. The medium was finally dispensed in different culture vials which were then tightly plugged with sterilized cotton. It was then autoclaved at 15-20 pounds/inch pressure at 121ºC for 20 minutes and then allowed to cool.

Table-5-Stock solutions of the different constituents of MS (1962) medium

| Stock solution code (MSSS-01) | | | Major Salts [strength =10x] | |
|---|---|---|---|---|
| Constituents | Quantity 9mg/L) in original medium | Quantity dissolved in stock solution | Final volume of the stock solution | Quantity to be used for prepration of one Litre MS medium |
| $NH_4NO_3$ | 1650 | 16.5g | 1000 ml | 100ml |
| $KNO_3$ | 1900 | 19.0g | | |
| $Cacl_2.2H_2O$ | 440 | 04.4g | | |
| $MgSO_4.7H_2O$ | 370 | 03.7g | | |
| $KH_2PO_4$ | 170 | 01.7g | | |
| Stock Solution Code (MSSS-02) | | | Minor Salts [strength =100x] | |
| KI | 0.83 | 08.3g | 500ml | 5.0ml |
| $H_2BO_3$ | 6.20 | 62.0g | | |
| $MnSO_4.4H_2O$ | 22.30 | 2230g | | |
| $ZnSO_4.7H_2O$ | 8.60 | 8.60g | | |
| $Na_2MoO_4.2H_2O$ | 0.250 | 0.25g | | |
| $CuSO_4.5H_2O$ | 0.025 | 0.025g | | |
| $CoCl_2.6H_2O$ | 0.025 | 0.025g | | |
| Stock Solution Code (MSSS-03) | | | Iron Source [strength=100x] | |
| $FeSO_4.7H_2O$ | 27.8 | 2.78g | 500ml | 5.0ml |
| $Na_2EDTA.2H_2O$ | 37.3 | 3.73g | | |
| Stock Solution Code (MSSS-04) | | | Myo-Inostol [strength=50x] | |
| Myo-inositol | 27.8 | 2.78g | 250ml | 5.0ml |
| Stock Solution Code (MSSS-05) | | | Organic ingredients [strength=100x] | |

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| | | | | |
|---|---|---|---|---|
| Thiamine HCl ($B_1$) | 0.1 | 10.0mg | 500ml | 5.0ml |
| Nicotinic acid ($B_5$) | 0.5 | 50.0mg | | |
| Pyridoxine HCl ($B_6$) | 0.5 | 50.0mg | | |
| Glycine | 2.0 | 200.0mg | | |

Inoculation:-Sterilized explants were inoculated onto aseptic basal medium (control) and phytohormone supplemented medium on the hood of laminar air flow chamber. The culture vials were then placed in incubation room under cool fluorescent illumination.

Culture Conditions and Care during Incubation:-Maintenance of constant environmental conditions throughout incubation is important. It was seen that continuous exposure of apple explants to light for more than three days reduces their growth potential and survival. Thus 12-l7hour light followed by 8-12 hour darkness was found effective for inducing the growth in most apple explants. However, seeds, embryos and tigella showed response only when kept in total darkness for at least 48 hours. Thus they were incubated in the vials wrapped with black paper or tin foil for Erst two days. Throughout experiment, incubation room was kept completely aseptic and most explants were cultured under cool white light with 1500-3000 Lux light intensity. Temperature of the room was maintained between 22-280C with 60-70% relative humidity. Shoot apices, axillary buds and seeds of apple often possess hidden contaminants. Their impact was controlled by immediately discarding the basal part of the incubated explants followed by dipping the cut end of their healthy parts in ethanol (for five seconds) during their transfer onto a new medium of same composition.

Control of Oxidative Browning:-The biggest problem with the culture of apple is the exudation of phenolic compounds by its explants (through their cut ends) especially those from mature trees which not only, after leaching out, turn medium brown but being deleterious also kill the explants. Following recommended techniques were tried:- (i) Use of PVP (Polyvinylpyrrolidon) 200-1000mg L4 in the medium (Walky, 1972) (ii) Use of ascorbic acid 50-100mg$L^{-1}$ (Lee and de Fossard, 1975,77) (Skirvin and Chu, 1977). (iii) Keeping cultures initially in dark for first few days (Monaco et a1, 1977). (iv) Use of cysteine HC1 (100mg $L^{-1}$) in the medium (Skirvin and Sharp, 1977) (v) Use of liquid nutrient media at 15°C for three days in continuous light (500Lux) and then transfer onto semisolid medium (Zimmerman, 1978). (vi) Use of activated charcoal 3.0g$L^{-1}$ (Reynolds and Murashige, 1979). (vii) Regular transfer of explants onto fresh nutrient medium of same composition for few days (Conger 1987, Hu and Wang, 1983). (viii) Addition of 8~hydroxyquinol sulphate (8-HQS 200ml of 0.1%solution (Machado et a1, 1991). (ix) Sealing of cut ends of explants with wax prior to inoculation (Bhat and Chandal, 1991). (x) Use of forced shoot tip culture method (Dalal et al 1992, 2006). (xi) Treatment of explants with anti-oxidants like citric acid 150mg$L^{-1}$ prior to inoculation (Kumar and Kumar, 1996). (xii) Rinsing sterilized explants with Diethyl-dithiocarbonate (DIECA 2g$L^{-1}$) prior to inoculation (Kumar and Kumar, 1996). While applying these techniques at least five times, poor results were obtained. Thus a modified technique (Rizvi et al. 2007, 09) was ultimately developed and followed which gave cent percent results. It involved:- (a) Selection of well irrigated trees as source of explants or using forced bud sprouts obtained by chilling branch cuttings of mature trees overnight and then placing them vertically in water containing Kn (lS-ZOµM) until buds sprouted. (b) Chilling of explants overnight (24hrs) in KN (15-20µM). (c) Reduction in the strength of MS major and minor salts in nutrient medium to half. (d) Regular transfer of explants onto fresh nutrient medium of same composition at least three to five times during first few days. (e) Addition of PG (10µM) in nutrient medium.

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Subculture:-Subculture was carried out on the hood of Laminar air flow chamber under aseptic conditions after every 4-6 weeks depending upon the organogenetic and proliferation potential of the explants. The products of explants were carefully separated out and inoculated in separate vials.

Regular Observation and Data Recording:-The cultures were daily monitored for contamination and growth. The changes in explant were recorded on weekly basis and the data was put in proper sequence and in tabulated form. It was also transcribed at the end of every week and stored as e-content.

Data Analysis and Interpretation:-After recording correct and accurate data about nature of media used, phytohormonal concentration, date of inoculation, incubation and subculture, nature of light i.e., its intensity, quality and duration, humidity etc. and their impact on explant response, callus growth, organogenesis, embryogenesis, it was analysed through statistical and mathematical methods. Ten / twenty replicates were taken for each treatment and observations were recorded at the end of every week. Analysis of variance (ANOVA) was carried to determine the significance of the results using Dunkan's multiple range test (a S 0.05) for mean number of shoots/ roots produced.

Photography:-To authenticate the research work, photography was done carefully and sequentially with the help of 8.2 megapixel digital camera. The digital photos were kept in a safe external hard drive in separate folders. Each folder was named after the year month and day of taking photographs. For example, photographs taken on 14th July 2006 were kept in a folder named 2006-07-14.

Hardening:-The plantlets obtained from different explants through repeated subcultures were finally left in culture vials with open mouth for three days in the incubation room, transferred to thumb pots containing peat-vermiculite or soil-peat mixture and then taken out of incubation room of the lab. Attempts were made to acclimatize plants under laboratory conditions as the green house facility could not be availed due to its late installation in the campus.

## III. RESULTS

Studies on Apple Culture:-The present research work represents an extension of M. Phil. work which was restricted to preliminary lab studies on two cultivars namely Ambri and Golden Delicious only. This work has now been extended to four apple cultivars namely Ambri, Chambura, Maharaji and Golden Delicious. The work was undertaken with the aim of investigating in vitro potential of different explants from mature trees as well as from seedlings using different plant growth regulators. Perusal of literature shows that the technique has the potential for growing apple trees in the valley on industrial scale as the conventional methods of propagation through grafting of scion cultivars onto root stocks and budding are not only time consuming but are also inefficient and insufficient.

Response of Source Material (Trees):-Thorough study on the selection process revealed that explants chosen from well irrigated trees yielded better results than those chosen from less irrigated trees. It was also observed that trees lying near brooks provided better source material than those lying in high slopes. The explants taken from irrigated trees and those near brooks released very less phenolic exudations and thus showed higher survival rate and greater power of morphogenesis which is evinced by the results depicted in Table 06.

Response of Explants:-Explants selected from the young twigs of 40-50 year old trees and taken during early morning showed far better results than those taken during mid-day time and in evening hours. The survival rate of the eXplants was also better (more than 90%). Shoot apices from juvenile branches yielded better results than those from mature branches. Dormant buds from mature branches were forced to sprout in the laboratory on their twigs kept in vertical position with their basal ends dipped in water containing kinetin (20μM)

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Table -6-Impact of irrigation on source material for the phenolic exudation. Survival rate and establishment power

| Explant | Well irrigated trees* | | | Less irrigated trees* | | |
|---|---|---|---|---|---|---|
| | Phenolic exudation | Survival rate[b] | Establishment Power[c] | Phenolic exudation | Survival rate[b] | Establishment Power[c] |
| Shoot Apices | + | 10% | 100% | +++ | 55% | 65% |
| Axillary Buds | + | 100% | 95% | +++ | 55% | 60% |
| Nodal Stem Segments | + | 100% | 90% | +++ | 45% | 60% |
| Leaf Segment | + | 100% | 85% | +++ | 66% | 60% |

+ -very less exudation (straw coloured), +++ - Heavy browning (dark brown) a -twenty replicates per treatment, b -percentage of explants survived, c -percentage of survived explants that expressed their totipotency

Standardization of Sterilization Procedure:- Shoot Apices and Axillary Buds:-Of different disinfectants used for the sterilization of shoot apices and axillary buds from mature trees of all the cultivars under reference, the best one was found to be mercuric chloride when used in two phases i.e. pre-chilling phase and post chilling phase. In pre-chilling phase 0.1% Mercuric chloride used for five minutes was found best whereas in post chilling phase 0.05% Mercuric chloride for 90 seconds was found to be best with 95% survival rate as depicted in Table 07.While using sodium hypochlorite, 90% sterilization with 45% survival rate was observed when 15% of it was applied during pre-chilling phase and 5% during post chilling phase. When ethanol was used in different concentrations a maximum of 35% survival rate with 40% contamination control could be registered.

Table-7-Efficacy of different sterilants for surface sterilization ot shoot apices and axillary buds from mature trees of four selected apple cultivars

| Explant | Disinfectant | Strength % | | Duration (Minutes) | | Response Percentage[b] | |
|---|---|---|---|---|---|---|---|
| | | Pre chilling phase | Post chilling phase | Pre chilling phase | Post chilling phase | Sterilization | Survival |
| Shoot apices and axillary buds | Mercuric chloride | 2.0 | 1.0 | 05 | 1.5 | 100 | Zero |
| | | 1.0 | 0.5 | 05 | 1.5 | 90 | 10 |
| | | 0.5 | 0.1 | 05 | 1.5 | 90 | 25 |
| | | 0.1 | 0.05 | 05 | 1.5 | 100 | 95 |
| | Sodium Hypochlorite | 25 | 15 | 05 | 3.0 | 100 | Zero |
| | | 20 | 10 | 05 | 3.0 | 90 | 10 |
| | | 15 | 05 | 05 | 3.0 | 30 | 45 |
| | | 5.0 | 01 | 05 | 3.0 | 30 | 55 |
| | Ethanol | 90 | 90 | 2.5 | 2.5 | 100 | Zero |
| | | 90 | 90 | 2.5 | 2.0 | 75 | Zero |
| | | 90 | 90 | 2.5 | 1.0 | 65 | 05 |
| | | 90 | 90 | 2.5 | 45sec | 40 | 35 |

Nodal Stems Segments:-Nodal stem segments obtained from mature trees of the four different cultivars showed best response (95%) in terms of survival rate and contamination control (100ºC) when they were disinfected with 0.1% Mercuric chloride for 7 minutes soon after their cutting from the trees in pre-chilling phase and for 90 seconds in post~chilling phase (Table 08). This was

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achieved only when they were cut out from the juvenile twigs (obtained from mature trees). Contamination could be controlled to the extent of 85% with 65% survival rate when sodium hypochlorite was applied in the concentrations of 15% and 500 for seven minutes and 90 seconds during pre-chilling and post chilling phases respectively.

Leaf Segments:-For the sterilization of leaf segments of all the four selected apple cultivars. the best results (with cent percent sterilization and survival rate) were obtained when juvenile leaves collected in early morning were first treated with Sodium hypochlorite (B 5%) for five minutes and then with Mercurie chloride (A 0.1%) for 90 seconds (Table 09) after chilling in kinetin (10-25µM) solution for 12-16 hours. The discs or segments from the aseptic leaves were finally inoculated onto nutrient medium.

Table-08-Efficacy of different sterilants for surface sterilization of nodal stem segments'of different apple cultivars (under reference)

| Explant | Disinfectant | Strength % | | Duration (Minutes) | | Response Percentage[b] | |
|---|---|---|---|---|---|---|---|
| | | Pre Chilling Phase | Post Chilling Phase | Pre Chilling Phase | Post Chilling Phase | Sterilization | Survival |
| Nodal stem Segment | Mercuric Chloride | 2.0 | 1.0 | 07 | 1.5 | 100 | Zero |
| | | 1.0 | 0.5 | 07 | 1.5 | 100 | 10 |
| | | 0.5 | 0.1 | 07 | 1.5 | 95 | 25 |
| | | 0.1 | 0.1 | 07 | 1.5 | 100 | 95 |
| | Sodium Hypo-chloride | 25 | 15 | 07 | 1.5 | 100 | Zero |
| | | 20 | 10 | 07 | 1.5 | 100 | 15 |
| | | 15 | 05 | 07 | 1.5 | 85 | 65 |
| | | 5.0 | 01 | 07 | 1.5 | 35 | 35 |

Seeds:-Seeds from mature trees responded best with cent percent survival rate when 0.1% Mercuric chloride was used for 10 minutes in first stage (before chilling) and 0.05% of it was used for five minutes in the second stage (after chilling at 4ºC in GA; for 7-14 days).The results are depicted in Table 10. Use of Sodium hypochlorite (5-25%) for 10 minutes in pre-chilling phase and for 5 minutes in post chilling phase could help in controlling contamination only to a maximum of 85% with 65 survival rate. Similarly when mercuric chloride (0.1 and 1.000) was used during pre~chilling phase followed by ethanol (90%) dip in post chilling phase, contamination could be controlled but survival rate did not exceed 65%.

Table-9-Efficacy of different sterilants for surface sterilization of leaf segments from mature trees[a] of the selected apple cultivars

| Explant | Disinfectant | Strength % | | Duration (Minutes) | | Response Percentage[b] | |
|---|---|---|---|---|---|---|---|
| | | Pre Chilling Phase | Post Chilling Phase | Pre Chilling Phase | Post Chilling Phase | Sterilization | Survival |
| Leaf segment | Mercuric Chloride (A) | 2.0 | 1.0 | 05 | 1.5 | 100 | Zero |
| | | 1.0 | 0.5 | 05 | 1.5 | 100 | 10 |
| | | 0.5 | 0.1 | 05 | 1.5 | 95 | 25 |
| | | 0.1 | 0.05 | 10 | 1.5 | 100 | 20 |
| | Sodium Hypochloride | 25 | 15 | 05 | 5.0 | 100 | Zero |
| | | 20 | 10 | 05 | 5.0 | 100 | 15 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (B) | 15 | 05 | 05 | 5.0 | 85 | 35 |
| | | 5.0 | 01 | 05 | 5.0 | 30 | 30 |
| | A+B | 25B | 1.0A | 05 | 1.5 | 100 | 10 |
| | | 20B | 0.5A | 05 | 1.5 | 100 | 25 |
| | | 15B | 0.1A | 05 | 1.5 | 100 | 65 |
| | | 5.0B | 0.1A | 05 | 1.5 | 100 | 100 |

Data recorded after four weeks –[b] -20 replicates per treatment [c] Sodium hypochlorite in pre-chilling phase and Mercuric chloride in post chilling phase

Table-10-Eficacy of different sterilants for surface sterilization of seeds obtained from mature fruits[a] of the selected apple cultivars

| Explant | Disinfectant | Strength % | | Duration (Minutes) | | Response Percentage[b] | |
|---|---|---|---|---|---|---|---|
| | | Pre Chilling Phase | Post Chilling Phase | Pre Chilling Phase | Post Chilling Phase | Sterilization | Survival |
| Seeds | Mercuric Chloride | 2.0 | 1.0 | 10 | 5.0 | 100 | Zero |
| | | 1.0 | 0.5 | 10 | 5.0 | 100 | 10 |
| | | 0.5 | 0.1 | 10 | 5.0 | 95 | 25 |
| | | 0.1 | 0.05 | 10 | 5.0 | 100 | 100 |
| | Sodium Hypochlorite | 25 | 15 | 10 | 5.0 | 85 | Zero |
| | | 20 | 10 | 10 | 5.0 | 85 | 15 |
| | | 15 | 05 | 10 | 5.0 | 75 | 65 |
| | | 5.0 | 01 | 10 | 5.0 | 30 | 65 |
| | Mercuric Chloride(A) +Ehanold (B) | 1.0A | 90B | 20 | 2.0 | 100 | Zero |
| | | 0.1A | 90B | 10 | 0.5 | Zero | 55 |
| | | 0.1A | 90B | 10 | 0.25 | 100 | 60 |

Date recorded after four weeks [b] -20 replicates per treatment

a maximum average number of 50 ± 1.35 shoots were produced per shoot per subculture (Table 12). The potential of shoot proliferation increased in the subcultures attempted. The level of significance of the results after applying ANOVA is depicted in tables 1 la and 12a.

Impact ofTDZ alone:-Use of TDZ did not yield successful results in case of MTSTs and only a maximum Variation in Phenolic Exudation and its Relationship with Explant Survival:-It was observed the rate of leaching of phenolic exudates varied in different cultivars when obtained from same place at same time with same environmental conditions, Ambri cultivar leached out more exudates than Chambura and Golden Delicious cultivars whereas Maharaji leached out least exudates. It was also noticed that rate of exudation of phenolic compounds shows inverse relation with the survival of the explants as depicted in Fig 01. Increased rate of leaching of phenolic exudates from the cut ends of the explants during incubation turned medium dark brown in which explants failed to show any response. Mature shoot tips obtained from less irrigated trees exuded maximum quantity of phenolic compounds as compared juvenile shoot tips from same trees. The inhibitory action of these exudates on physiological and metabolic activities of the explants led to their necrosis and death. However, such shoots showed some chance of survival only when they were regularly transferred on to fresh nutrient medium of same composition. The most interesting result was that if the

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explants obtained from mature trees were subjected to pre-inoculation chilling in Kn (lS-20µM) for 24hrs, rate of leaching of the phenolic exudates was reduced the to one fourth. However, over-chilling in the solution showed adverse effects due to anaerobic conditions.

Chambura Cultivar:- Shoot Apices From Mature Trees and in vitro Grown Seedlings:-

Culture Establishment and shoot multiplication:-Double sterilized and pre-chilled shoot apices of Chambura cultivar obtained from mature trees (MTSTs) and in vitro raised seedlings (SBSTs) were inoculated on MS medium augmented with BA, KN and TDZ with and without PC the results of which are summarised in tables 11 and 12. As in case of Ambri and Golden Delicious cultivars, little response was seen on MS medium with full strength of its salts but good results were recorded when the strength of the medium was reduced to half.

Impact of BA alone:-Under the influence of BA (0.5-1.5µM) the MTSTs (mature tree shoot tips) turned brown within 48 hours and then faced necrosis. Increase in BA concentration upto 3.5µM resulted in the formation of light yellow callus (LYC) at cut ends in about 35% shoo tips. Further increase in its concentration upto 5.0µM resulted in axillary shoot proliferation in about 40% shoots with a maximum of 14 ± 0.72 shoots per explant (Tablel l). SBSTS (seedling born shoot tips) of same cultivar developed light yellow loose callus (LYLC) when they were cultured on MS (x½) medium fortified with BA (2.0-3.5µM) and produced axillary and adventitious shoots when the same medium was supplemented with BA (4.0-5.0µM). However, only a maximum of 70% cultures developed multiple shoots with maximum average number of 24 ± 0.72 shoots per shoot per subculture (Table 12).

Impact of BA with PG:- Culture of MTSTs on MS (l/2) supplemented with BA and PG yielded good results. No response was seen when they were cultured under the influence of low cytokinin i.e. BA (O.5-2.0µM) + PG (10µM). Increase in BA concentration from 2.0 to 3.5µM resulted in the development of callus at cut ends in 25% shoots. Further increase in BA concentration from 4.0 to 5.0µM with PG (10µM) favoured growth of apical bud as well as axillary buds and their proliferation (ASP). The best reSponse was observed on MS (l/2) + BA (4µM) + PG (10µM). Unlike Ambri and Golden Delicious cultivars increase in BA concentration from 4.0 to 4.5 and 5.0µM decreased percentage of response from 90% to 70% and number of adventitious and axillary shoots from 42±0.7l to 36±0.82 per shoot as depicted in table 11. The shoots obtained were subcultured individually or in lumps several times to increase number of shoots. The potential of shoot proliferation continued in the subcultures attempted. SBSTs of same cultivar developed light yellow loose callus (LYLC) when the medium was fortified with BA (2.0-3.5µM)+ PG (10µM) but developed axillary and adventitious shoots when the medium was supplemented with BA (4.0-5.0µM)+ PG (10µM). Best (cent percent) re5ponse was seen on the medium containing BA (4.0µM) when of 30% shoots produced loose creamy callus (LCC) at cut ends (CCE). However, about 40% SBSTs showed axillary shoot proliferation on MS (1/2) + TDZ (4µM) + PG (10µM) and produced a maximum average number of 40:20.72 shoots per subculture (Table l l).

Impact of TDZ with PG:- Addition of PG to the medium containing TDZ did not help in getting successful results during the culture of MTSTs as no response was seen while using 0.5-2.5µM TDZ with PG (lOµM). Loose creamy callus (LCC) at cut ends (CCE) was observed when concentration of TDZ was increased to 3.0 and 3.5µM with PG (10µM). However, 25-30% shoots showed axillary shoot proliferation (ASP) with further increase in the concentration of TDZ to 4.0, 4.5 and 5.0µM with PG (10µM). The number of shoots produced was 14±2.11, 12±0.32 and 14±0.45 respectively (Table 11). When SBSTs of same cultivar were cultured under the influence of TDZ+PG callus formation was observed under low concentrations of TDZ (3.0 and 3.5 µM) and axillary shoot proliferation was seen in 45% shoots (Table 12).

Axillary Buds From Mature Trees and in vitro Grown Seedlings:-Axillary bud sprouts obtained from pre-sterilized stem cuttings of mature Chambura trees (MTABs) and in vitro raised seedlings (SBABs) of Chambura apple cultivar were cultured under the influence of BA alone, BA with PG, TDZ alone and TDZ with PG (Tables 13 and 14). MTABs established Well on MS (X ½)

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medium augmented with BA (2-5µM) + PG (10µM) but developed multiple shoots only when the same medium was augmented with BA (4-5µM) + PG (10µM). The buds started sprouting in the fourth week of culture period on MS (X ½) + BA (4µM) + PG (l0µM) (Plate-XXVII, Fig.01) with the survival rate of 90%. The multiple shoots produced were subjected to subculturing several times to increase their number. The average shoot number per subculture was recorded to be 30± 0.72. Tiny SBABs also proliferated well on same medium augmented with same supplements (Table 14). Best response was seen under the influence of BA (4.0µM) + PG (10µM). Proliferation started at the end of second week of culture period followed by multiple adventitious shoot formation from the basal ends of the buds. The shoots produced repeatedly subcultured at the interval of eight weeks to increase their number. The average shoot number per subculture was found to be 34 ± 0.78. The level of significance of the results after applying ANOVA is depicted in tables 13a and 14a. Nodal Stem Segments from Mature Trees and in vitro Grown Seedlings Nodal stem segments obtained from juvenile branches of mature Golden Delicious trees (MTNS) and in vitro grown seedlings (SBNS) were inoculated on MS (X½) supplemented with BA or KN with or without PG the results of which are depicted in tables 15 and 16. About 35% MTNS developed moderate loose yellow callus (MYLC) at cut ends when the medium was fortified with BA (3.0-4.0µM). 1500 explants developed 12i0.88 multiple adventitious shoots at basal end. When KN (5.0µM) was used in the medium a maximum of 20% explants developed callus at cut ends. Best results were achieved when MTNS were placed on MS (X ½) + BA (5µM) + PG (10µM) as in previous cases. The explants developed multiple adventitious shoots at basal ends in five weeks. The shoot number was increased by subculturing them on same medium to 14 ±1.85 /shoot. Nodal explants obtained from in vitro grown seedlings (SBNS) of same cultivar started multiple shoot formation just in third week of the culture on the same medium with same supplements. The adventitious shoots thus produced were repeatedly subcultured to increase their number. The potential of shoot proliferation increased with the increase in the number of subcultures. The maximum average number of shoots produced per subculture per shoot was found to be 30±0.65. The level of significance of the results after applying ANOVA is depicted in tables' l6a and 16a.

Leaf discs/segments from mature trees and in vitro Grown Seedlings:-Creamy white loose callus was produced along the cut edges of leaf segments obtained from mature trees (MTLS) of Chambura cultivar when they were cultured on MS (X½) medium under the influence of KN or BA (2.0-3.5µM) (Table 17). Expansion accompanied by callus formation along cut edges was also noticed when the explants were cultured in presence of $GA_3$ (4µM). Multiple adventitious shoots were formed along cut edges of the same leaf segments after about four weeks of culture period when they were placed on MS (X ½)+BA (4-5µM)+PG (10µM). Both callus and the adventitious shoots produced were subcultured many times separately on same medium for six months. Similar results were shown by the in vitro raised leaf segments (SBLS) of same cultivar when they were cultured on MS medium containing different adjuvants (Table 18). When cultured on MS (X ½) + $GA_3$ (4µM) they expressed callogenenetic potential but on MS (X ½) + BA (4µM) + PG (l0µM) they expressed organogentic potential by way of direct multiple adventitious shoot formation along cut edges. The level of significance of the results after applying ANOVA is depicted in tables 17a and 18a.

Root-Shoot Transitional Regions:-RSTs of Chambura cultivar were cultured under the influence of different phytohormones with or without PG (Table 19). They developed callus at cut ends when cultured under the influence BA (2-4µM) + PG (10µM) in three to four weeks of culture period. The callus produced was tried for organogenesis after subculturing where shoots were produced (depicted in callus culture). The level of significance of the results after applying ANOVA is depicted in tables 19a.

Root Segments:-Callus formation was observed when the root segments of Chambura cultivar obtained through in vitro means were cultured on MS medium, containing $GA_3$ (2-5µM) but formation of adventitious buds was noticed on upper surface after three weeks when they were cultured on the medium augmented with a lower BA (2.5µM) concentration (Table 20). The buds were allowed to proliferate which later on produced multiple shoots.

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Seeds / Embryos:-Testa free Chambura seeds followed normal mode of germination on MS (XV2) medium supplemented with BA (4µM) + PG (l0µM) within 24 hours (Table 21a and 20b) . Cotyledons opened and root pole started elongation followed by shoot pole. However further growth in the root pole stopped but that in shoot pole continued for some time. It was followed by multiple shoot formation from cotyledonary node. All parts were isolated and subcultured separately. The shoots developed multiple adventitious shoots at its base. The shoots were multiplied through repeated subcultures on same medium with same supplements until sufficient number of shoots was produced. About 45i0.76 shoots were produced per shoot per subculture.

Tigellum:-Elongation in hypocotyl as well as epicotyl was seen in 12i2 hours when tigella excised from Chambura seeds were cultured under the influence of BA (5.0µM)+IBA (2.5µM). However, further growth could not continue. When same tigella were cultured on BA (2.5-5µM) with PG (l0µM), shoot pole showed rapid growth which was immediately followed by growth of axillary buds. The shoots obtained were subcultured several times and then subjected to rooting.

Cotyledons:-Cotyledons obtained from the seeds of Chambura cultivar produced white loose callus after three weeks of culture period and then formed adventitious roots when they were cultured on MS (X'/2) medium fortified with IAA (1.0µM) + IBA (1.0µM) + NAA (1.0µM). On MS (X ½) + BA (1.0µM) + PG (10µM) they turned green and produced callus at base but developed multiple adventitious shoots from margins (Plate XXXIV, Fig.01). The shoots proliferated well and attained a considerable length of 3.5i0.45cm in eight Weeks of culture period. The shoots obtained were subcultured on MS (X½) + BA (4.0µM) + PG (l0µM) for further multiplication.

Callus:- Callus was obtained from shoot tips, axillary buds, nodal stem segments, leaf segments. Embryos. Cotyledons. Tigellum and root segments of Chambura 61 cultivar under the inlluence of $GA_3$, auxins and cytokinins (Table 23). The callus formed from the mature tree explants was light yellow and very loose and turned dark brown in about six weeks. That formed from the seedling explants was initially white and loose but turned green and compact in about six weeks. Calluses obtained from mature tree explants and seedling explants were separately placed on MS medium to study their response to different phytohonnones like BA, KN, GA3, IBA, IAA, NAA and TDZ etc (Table 23). It was seen that the callus differentiated into adventitious roots under the influence of [BA and into shoots under the influence of BA but developed embryoids under the influence of $GA_3$. It was also observed that the callus obtained from seedling explants has very high organogenetic potential than that produced from mature tree explants.

Rooting of in vitro raised Shoots:-The shoots obtained through the mature tree explant culture (MTEC) and seedling born explant culture (SBEC) were subcultured onto rooting medium containing auxins like IBA, IAA, NAA as summarised in tables 24 and 25 and is detailed below.

Impact of IAA, NAA and IBA+IAA+NAA:-When the microshoots obtained through MTEC were cultured under the influence of IAA or NAA no response was seen until their concentration was increased to 3.0µM while callus formation was observed at cut ends (CCE) of microshoots at 3.0-5.0µM concentration in about 30% shoots. The callus produced was creamy yellow and loose (CYC). Rooting could not be achieved with the help of IAA and NAA alone or with BA (Table 24). Similar response was shown by the microshoots obtained through SBEC (Table 25).

Impact of IBA + IAA + NAA:-When shoot apices of the same selected cultivar obtained through MTSTC and SBSTC were cultured on MS(X ½) supplemented with IBA+IAA+NAA (2.0-5.0µM), they produced callus at cut ends (CCE). The callus produced by the shoots obtained through MTEC was loose light brown massive callus (LLMC) while that produced by those obtained through SBEC was loose and light yellow (LYLC) (Tables 24 and 25).

Impact of IBA without PG:-Use of 1.0 and 1.5µM IBA alone promoted callus formation at basal end in about 5% shoots. The callus produced was creamy yellow (CYC). Increase in its concentration to 2.0 and 2.5µM resulted in development of adventitious

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roots in about 60% plants obtained through MTEC with an average of 14±0.82 roots per shoot (Table 24). The shoots obtained through SBEC also showed similar response. About 85% shoots produced adventitious roots with an average of 12±0.82 roots per shoot when the medium was fortified with IBA (2.5µM) (Table 25).

Impact of IBA with PG:-No response was seen when the Chambura shoots obtained through MTEC were cultured in presence of 0.5, 1.0 and 1.5µM IBA with PG (10µM) whereas callose roots were produced when the concentration of IBA was increased to 2.0µM. Further increase in the concentration of IBA to 2.5µM resulted in the initiation and development of adventitious roots in all shoots (100% response) with an average of 12 ±:0.8l roots per shoot. Microshoots of Chambura apple cultivar obtained through SBEC cultured under the influence of IBA (0.5. 1.0 and 1.5µM) with PG (10µM) did not show any response and produced callose roots when the medium was fortified with IBA (2.0µM) + PG (10µM). When the concentration of IBA was increased further to 2.5µM the microshoots produced adventitious roots (with cent percent response) having an average number of 8 ± 0.65 roots per microshoot. Further increase in the concentration of IBA to 3.0, 3.5, 4.0, 4.Sand 5.0µM favoured formation of callose roots but with decreased response. The level of significance of the results after applying ANOVA is depicted in tables 24a and 25a.

Hardening of the microplants:-The microplants produced through shoot tip culture were taken out of culture vials very carefully, roots were washed under running water to remove agar and then they were transferred to pots containing soil mix (soil peat ltl). Potted plants were covered by perforated polyethene bags and kept under continuous observation. The plants were misted after regular intervals to maintain maximum humidity (90-00%) under laboratory conditions. The survival rate was found to be 80% in the plants obtained through MTEC and 95% in those obtained through SBEC.

Establishment of Protocol:-From aforementioned observations it can be envisaged that the establishment of the explants from mature trees (MTEs) and seedling born explants (SBEs) of chambura cultivar occurs best on MS (X½) + BA (4.05.0µM) + PG (l0µM), shoot multiplication occurs best on same medium augmented with BA (4.0µM) + PG (l0µM) and root induction occurs best on the medium containing lBA. An outline of the complete protocol of shoot tip culture of Chambura cultivar of apple obtained from mature trees has been summarised in plate XXXV for its clonal propagation.

Culture Establishment:-0.5-1.0cm long shoot tips of Maharaji cultivar, collected from 40-50 year old mature trees (MTSTs), were initially inoculated MS basal medium. No response was seen as the tips turned and necrosed. When the salt strength of the medium was reduced to half, it was seen that browning and death process delayed but proliferation could not be achieved. The medium with half salt strength supplemented with BA alone, BA with PG, TDZ alone and TDZ with PG was then used (Table 11). Best response in terms of shoot proliferation with high survival rate (95%) was achieved when the medium was fortified with BA (4µM) + PG (10pM). The shoot tips got well established in five weeks. Similar response was shown by the shoot tips obtained from in vitro raised seedlings (SBSTs) of same cultivar. However survival rate was found to be 100% and the shoots got well established only in two weeks time (Table 12).

Shoot Multiplication:-Once shoots tips from the mature trees (STMTs) and in vitro raised seedlings (SBSTs) of Maharaji cultivar got fully established, possibility for the exploitation of their multiplication potential was explored by using BA and TDZ with and without PG (Tables 11 and 12).

Response of shoot tips to BA alone:-Addition of BA alone to the culture medium could not provide any significant results in both MTSTs and SBSTs. When BA (0.5, 1.0 and 1.5µM) was added to the MS (X½) medium MTSTs withered and finally died. Increase in its concentration from 2.0 to 3.5µM could help in the development of callus at basal end in about 35% shoots. The callus produced was loose and light yellow (LYC). Further increase in its concentration to 5.0µM helped in the induction of axillary shoot proliferation in about 45% shoot tips with 16 ± 0.82 shoots per subculture (Table ll). SBSTs also showed poor response to the medium augmented with BA alone. BA (2.0-3.5µM) induced formation of light yellow loose callus (LYLC) at cut

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ends. Higher BA (4.0-5.0µM) concentration promoted axillary shoot proliferation (ASP) with cent per cent response and a maximum of 28 ± 058 shoots per subculture were registered (Table 12).

Response of shoot tips to BA with PC:-Shoot tips of same cultivar obtained from mature trees (MTSTs) did not show any rcsponse when the medium was augmented with BA (0.5, 1.0, 1.5 and 2.0µM) + PG (10µM). A maximum of 35% shoots developed a loose yellow callus (LYC) at cut end when the concentration of BA was increased to 2.5, 3.0 and 3.5µM. Further increase in its concentration to 4.0, 4.5 and 5.0µM favoured multiple axillary shoot proliferation (Table 11). On MS (X ½) + BA (4µM) + PG (l0µM) multiple shoot formation at basal ends started in fifth week. The shoots produced were subcultured on same medium to increase their number. The maximum average number of shoots produced per subculture was found to be 48120.82 highest in all cultivars tried. The established shoots tips obtained from in vitro raised seedlings (SBSTs) of same cultivar also responded best when the medium was supplemented with BA (4µM) +PG (l0µM). The cut end of the explants developed adventitious shoots after about 20 days of incubation. The shoots produced were multiplied through repeated subcultures as in other cultivars. The average number of shoots produced per explant per subculture was recorded to be 52:1:1.75 again highest in the cultivars tried. The level of significance of the results after applying ANOVA is depicted in tables 1 la and 12a.

Response of shoot tips to TDZ alone:-No response was shown by MTSTs when the shoot tips were cultured on MS (X½) medium augmented with 0.5, 1.0 and 1.5µM TDZ. Increase in the concentration of TDZ from 2.0 to 5.0pM resulted in the in the formation of loose creamy callus (LCC) at cut ends. Multiple shoot formation could not be seen (Table l l). SBSTs of same cultivar also produced callus at basal end on the medium fortified with TDZ (2.0 to 3.5µM). Further increase in the concentration of TDZ upto 5.0µM resulted in development of adventitious shoot formation in about 45% shoots with an average of 40 ± 0.78 shoots per subculture (Table 12).

Response of shoot tips to TDZ with PC:-The combination of TDZ with PG (10µM) did not yield any significant results in case of MTSTs. No response was seen on the medium augmented with 0.5-2.5µM TDZ and PG (10µM). A maximum of 15% shoot tips developed loose creamy callus (LCC) when the concentration of TDZ was increased to 3.0 and 3.5µM. Further increase in the concentration of TDZ to 4.0, 4.5 and 5.0 helped in the induction of axillary shoot proliferation in about 35% shoot tips. The shoot number produced per shoot tip was found to be SBSTS also produced callus when TDZ (0.5-3.5µM) used and showed axillary shoot proliferation when it was used in higher concentration (4.0 -5.0µM). The maximum average shoot number produced per subculture was found to be 32:0.75 (Table 12).

Axillary Buds From Mature Trees and in vitro Grown Seedlings:- Axillary bud sprouts obtained from the young twigs of 40-50 year old trees (MTABs) and from in vitro raised seedlings (SBABs) of Maharaji cultivar were inoculated on MS medium containing cytokinins and TDZ with and without PG (Table 13 and 14). The sprouts obtained from mature trees (MTABs) established best on MS(X ½) medium augmented with BA (4µM) + PG (10µM) with 95% survival rate. It was followed by multiple shoot formation after four weeks of culture period. The shoots were subcultured many times on same nutrient medium with same phytohorrnonal concentration. The average shoot number per explant per culture was found to be 3l ± 0.80 (Table 13). When the buds of same cultivar were cultured on MS (X ½) + BA (23.5µM) they produced light yellow and loose callus (LYLC) at cut ends (Table 13). When TDZ was used instead of BA no response was noted upto a concentration of 1.5µM. However, callus was produced at a cone of 2.0-3.5µM and axillary shoots developed in 30% shoots between 4.0-5.0µM concentrations with average of 25 ±0.87 shoots per axillary bud per subculture (Table 13). Axillary buds obtained from Maharaji seedlings (SBABs) also started proliferation and multiple shoot formation at the end of second week of culture period When they were cultured on MS (X½) + BA (4.0µM) + PG (l0µM). Highest number of adventitious shoots was seen here among all cultivars with an average of 362 ± 0.66 shoots/ culture/explant (Table 14). The level of significance of the results after applying ANOVA is depicted in tables

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13A and 14a.

Nodal Stem Segments From Mature Trees and in vitro Grown Seedlings:-Nodal stem segments from mature trees (MTNS) of Maharaji apple cultivar developed moderate yellow loose callus (MYLC) at cut ends when they were cultured on MS (X ½) medium under the influence of BA (3.0-4.0µM) or KN (5.0µM) and initiated intense brown loose callus (BLC) when the same medium was fortified with Kn(5.0 µM) + PG(l0µM) or BA (5.0 µM) + KN (5.0µM) + PG ( I0µM) as depicted in Table 15. Best response was seen on MS (X1/2) medium augmented with BA (45µM) + PG (I0µM) in terms of multiple adventitious shoot formation in 85% explants. The shoots regenerated were isolated subcultured and subjected to rooting depicted separately. Nodal stem segments taken from seedling explants (SBNS) of same cultivar produced medium green compact callus (MGCC) when MS(X′/2) medium was augmented with BA(3-4 µM) and high green compact callus (HGCC) when KN (4-5µM) was used with PG (10µM) (Table 16). However, when they were cultured on MS(X ½) + BA (5µM) + PG (l0µM), these started showing multiple shoot formation just in third week of culture period. The adventitious shoots produced were repeatedly subcultured on same medium with same enrichments to increase their number. The level of significance of the results after applying ANOVA is depicted in Tables' l5a and 16a.

Leaf discs I segments from mature trees and in vitro Grown Seedlings:-Leaf segments from the mature trees (MTLS) of Maharaji apple cultivar developed callus along the cut ends when the medium was supplemented with $GA_3$(4µM) or BA (4 µM) (Table 17). The callus was creamy light yellow and loose and turned brown after three months. However, when the medium was augmented with BA (4µM) + PG (10µM), multiple adventitious shoots were produced along cut edges. The shoots were subcultured individually or in lumps several times to increase their number. Then they were transferred to rooting medium.

Leaf segments of same cultivar obtained from in vitro born seedlings (SBLS) when cultured on MS (X ½) + $GA_3$(4 µM), they produced compact green nodular callus (Table 18). But when they were cultured on MS(X ½) + BA (4µM) + PG (10µM) these showed direct multiple adventitious shoot formation. The level of significance of the results after applying ANOVA is depicted inTables 17a and 18a.

Root-Shoot Transitional Regions:-Callus formation was also observed at both the cut ends when RSTRs of Maharaji cultivar were cultured under the influence of BA (5pM) + IBA (2.5µM) within three weeks of culture period (Table 19). The callus was later on treated for exploiting the organogenetic potential (details in callus culture). The level of significance of the results after applying ANOVA is depicted in tables 1921.

Root Segments:-Root segments obtained from in vitra raised plants of Maharaji cultivar were cultured on MS medium containing different media supplements (Table 20). Best response in terms of caulogenesis was recorded on the medium containing lower BA concentration (2.5µM) with PG (10). The response was scored by way of forming adventitious buds on upper surface after three weeks of culture period. The buds produced were subcultured on the medium with same supplements which after shoot formation were subjected to rooting.

Seeds / Embryos:-Chilled, partially germinated, double sterilized and testa free seeds of Maharaji cultivar showed rapid germination on MS (X½) + BA (4µM) + PG (10µM) within 12 hours after inoculation (Tables 21a and 20b). The growth was initially normal but got arrested in root pole after some time. Shoot pole first produced single shoot which was followed by multiple shoot formation in second week of culture period. Shoot proliferation continued and the number increased to maximum average of 462±0.66 per explant in third week of culture period. In 10% seeds, shoot pole directly produced multiple shoots soon after inoculation. The shoots were subcultured repeatedly to increase their number and then transferred onto rooting medium.

Tigellum:-Tigella obtained from Maharaji seeds showed vigorous growth in shoot pole when cultured on BA (2.5-5µM) with PG

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(10µM (Table 22). Simultaneous growth of apical as well as lateral buds was observed when they were cultured on the medium containing BA (5µM) +PG (l0µM). Multiple shoots produced through repeated Subculture on same medium were then subjected to rooting.

Cotyledons:-Rapid expansion was seen in the isolated cotyledons of Maharaji cultivar on MS (X½) medium containing BA (l.0µM) +PG (10µM). The green cotyledons produced callus at cut ends in two weeks of culture period. Adventitious shoot formation along margins was seen in three months of culture period. As in other cultivars the shoots were subcultured repeatedly until their number increased in multiples. Then they were subjected to rooting on the medium.

Callus Differentiation:-Both mature tree explants and seedling explants of Maharaja apple cultivar yielded profuse callus when they were cultured under the influence of IBA (1.0-3.5µM), NAA (3.0-5.0µM), IBA+NAA+IAA (2.0-5.0µM) and TDZ (2.0-3.5µM) (Table 23). The callus obtained from seedling explants was initially white and loose but turned green and compact in about six weeks. That obtained from the mature tree explants was initially loose and creamy white or light yellow but turned brown in six weeks. The organogenetic potential of the callus obtained from seedling explants was very high. Multiple adventitious shoots were formed when the calli obtained from different seedling explants were cultured on the medium enriched with BA (5.0µM) +PG (10µM) with average number of 50 ± 0.88 per subculture (Table 23).

Rooting of Shoots:-The shoots produced by the explants of Maharaji cultivar obtained from lnature trees (MTES) and in vitro born seedlings (SBES) were subcultured individually on rooting medium containing auxins with or without PG to induce rhizogenesis (Tables 24 and 25)..

Impact of IAA on Rooting:-When MS (X ½) medium was supplemented with IAA (0.5-3.5µM) no rooting or callus formation was seen. However, shoot growth stopped and leaves started falling off from the shoots. Higher concentration of IAA (4.0-5.0µM) induced callus at cut end in 30% shoots. Rooting could not be registered.

Impact of NAA on Rooting:-Addition of NAA (0.5-2.5µM) to MS (X ½) medium could not help in achieving any significant desired result in the shoots. However, its higher concentrations (3.0-5.0 µM) induced callus formation in 30% shoot. Rooting was not registered at all.

Impact of IBA+IAA+NAA on Rooting:-A triple combination of auxins (IBA+IAA+ NAA) could only help in production loose, light brown massive callus (LLMC) at cut ends of the shoots with cent percent response at 2.5pM concentration. However, rooting response could not be achieved.

Impact of IBA without PG on Rooting:-Use of 1.0 and 1.5µM IBA without PG induced callus formation at the basal end of shoots obtained through MTE culture (MTEC). The callus produced was creamy yellow and loose (CYC). Its higher concentration (2.0 and 2.5µM) favoured normal rooting in 80% shoots with an average of 143 ±0.66 roots per shoot. Further increase in its concentration upto 5.0 µM resulted in the production of callose roots. Similar response was shown by the shoots obtained through SBE culture (SBEC). Lower IBA concentration (1.0 and l.5µM) induced callus formation at basal ends, moderate concentration (2.0 and 2.5µM) induced adventitious root formation and high IBA concentration (3.0 and 5.0µM) favoured formation of callose roots in 85% shoots (Table 25).

Impact oleA with PC on Rooting:-Normal rooting with cent percent response was observed in the shoots obtained through MTEC when the medium was augmented with IBA (2.5µM) + PG (l0µM). Rooting started in the second week of subculture and complete plantlets worth transplantation were obtained in six weeks of culture period. Higher concentration of IBA (3.0-5.0µM) with PG (10µM) could only yield callose roots in 70% shoots. Shoots obtained through SBEC also produced callose roots when the medium was augmented with low IBA (2.0µM) and its high (3.0-5.0µM) concentrations. Normal roots were produced only when IBA (2.5µM) was used with PG (10µM) (Table 25). The average number of roots produced was 8i0.88 roots per shoot. The level

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of significance of the results after applying ANOVA is depicted in tables 24a and 25a.

Hardening of the microplants:-Being a highly difficult task, extreme care was taken for hardening of the plantlets formed through the culture of different explants from mature trees (MTEC) and seedling born explants (SBEC). Four to six week old microplants were carefully taken out of culture vials and tubes their roots were washed under running water to remove agar and then they were transferred to thumb pots containing peat soil mixture in the ratio l: 1. The thumb pots were covered by polyethcne bags having perforations. High humidity (90-100%) and moderate temperature (25 ±2°C) were maintained under laboratory conditions to prevent the microplants from wilting. The survival rate under laboratory conditions was found to be 85%

Establishment of Protocol:-Culture of different exlants from mature trees (MTEC) and from in vitro raised seedling (SBEC) of Maharaji cultivar revealed that establishment and shool multiplication occurs best on MS (X ½ ) + (BA 4µM) + PG (10µM) medium and successful rooting occurs on MS (X½) +IBA (2.5µM) + PG (10µM). Microplants obtained though in vitro means get hardened well on soil and peat mixture in the ratio 1:1 when transplanted 4-6 weeks after rooting. The transplanted plants need to be humidified after regular intervals and left under high light intensity and moderate (25±2°C) temperature. It was also observed that shoot apex culture from mature trees is best suited for commercial purposes to avoid grafting involving stock-scion union for clonal propagation. The complete procedure of shoot apex culture from mature trees of Maharaji cultivar of apple has thus been summarized.

## IV. DISCUSSION AND CONCLUSION

Although tissue culture work on apple was started more than half a century ago (Letham, 1958) and nearly 360 research papers have been published, some aspects have not yet been touched and many aspects need to be reinvestigated. Having more than 8000 different varieties / cultivars, 330 of which have been reported in Kashmir valley alone in around 1972, less than 100 have been cultured through in vitro means using different explants in different tissue culture centres of the world. Present investigations on Ambri, Golden Delicious, Chambura and Maharaji cultivars of apple suggest that all explants cultured under the influence of different phytohormonal regimes have a very high potential for organogenesis either directly or through callus formation. By standardizing this technique, the big problem of regeneration of true to type apple plants in large numbers without grafting can be solved and thus can provide backbone for upliftment of apple industry in J&K state. It is also expected to serve in the conservation of apple cultivars endemic to Kashmir and on the way towards extinction. Several problems related to apple culture have been attended to but some are yet vexed. The problems which need special attention include: bacterial contamination in cultures even after several months of culture initiation, tissue vitrification, variability of response among different cultivars and root stocks, phenotypic stability of regenerated plants and cost of production (Kumar and Kumar, 1998). The success in the micropropagation of apple lies in the conditions in which parent plant survives, type of explant used, time of collection of explant, accuracy in sterilization procedure, composition of nutrient medium, type of hormones Used, control over oxidative browning after inoculation and hardening of plants produced. Present investigation on four cultivars of apple namely Ambri, Golden Delicious, Chambura and Maharaji have helped in solving a few of these problems which have been aimed at. Selection of mature apple trees as a source of explants constitutes one of the primary aspects in apple culture tlnough in vitro means. In present studies selection of explants from well irrigated trees especially those found near brooks was found to be highly effective over those selected from less irrigated trees. No such report has been published earlier. Zimmerman (1984) used dormant buds collected from the field after the rest period and directly disinfected them before inoculation. Dunstan (I984) collected dormant budwood in early autumn and isolated uninodal shoot pieces for the culture. A different technique in which green house plants were used as source material and 8-10cm long stem cuttings were cultured after sterilization has also been described (Srisikandaraja et al., 1982). Bhat et al. (2000) has found good irrigation of trees as essential aspect for successful culture of Red Delicious and American cultivars of apple. In a

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recent work dormant buds collected in different seasons from mature trees were forced to sprout on nutrient medium after sterilization (Dalal et al., 2006). The time of collection of explants represents the second important aspect in successful culture of apple. In present investigation explants collected in the early morning (between 5.30-8.00AM) showed far better results in terms of culture establishment than those collected during late morning, midday time or in afternoon period. This seems to be due the well hydrated condition of explants in the wee hours of morning as stomata remain closed at night. No such report has come to light till date. Kumar and Kumar (1998) have suggested that the explants collected from actively growing shoots should be collected in plastic bags to prevent wilting till further processing. When the plants are in dormancy, dormant shoots should be collected in early autumn and bud scales should be removed before sterilization (Zimmerman, 1984). Sriskandarajah et a1. (1982) suggest that explants should be collected from greenhouse plants. Being woody plants, apple trees remain fully loaded with contaminants and thus their explants are very difficult to sterilize fully as they contain hidden contaminants which appear even after several months of culture period. Different procedures have been suggested for the sterilization of apple explants. Detergents, wetting agents and sterilants have been used to achieve successful sterilization in the explants of different apple cultivars. Thorough washing of the apple explants in running tap water for half an hour using Laboline detergent and one or two drops Tween-20 or Mannoxol (0.01%) as wetting agent followed by sterilization in 0.1% $HgCl_2$ has been reported by many researchers (Nekrosova, 1964; Jones et al., 1977; Mehra and Sachdeva, l980, Sedlac et al., 2001; Shanna et al., 2004). Initial sterilization in $HgCl_2$ followed by overnight storage explants in liquid medium has also been found fruitful (Jones et al., 1977; Robinson and Schwabe, 1977). Better results have also been achieved by stirring shoot tips in basal medium for 20 minutes prior to inoculation (Zimmerman, 1984) and use of ethanol (70%) as sterilant (Zimmerman, 1985; Shanna et al., 2004). Sodium hypochlorite (5-20%) for 5-20 minutes has also been found effective in achieving complete sterilization in shoot tips and nodal stem segments (Dantas et al., 2006; Butiuc-Keul er al., 2010). Zimmerman (1984) has suggested use of calcium hypochlorite (20%) for achieving cent percent success and survival in many apple cultivars. In present investigation use of mercuric chlorite (0.100) for different time durations in prechilling and post chilling periods was found highly effective for sterilization of explants obtained from mature trees (Rizvi et al., 2007 and 2009). Browning of culture medium soon after the inoculation of explants (from mature trees) has remained a big hurdle for successful culture of apple in past. Several remedial measures have been suggested from time to time using in the medium PVP (Walky, 1972), ascorbic acid (Lee and de Fossard 1975, 77; Skirvin and Chu 1977), cysteine HCl (Skirvin and Sharp, 1977), activated charcoal (Reynolds and Murashige 1979), HQS (Machado et al., 1991), keeping cultures initially dark for first few days (Monaco et al., 1977), using liquid nutrient media in continuous light for initial period (Zimmerman, 1978), sealing of cut ends of explants with wax prior to inoculation (Bhat and Chandal, 1991). In Present research work, a remarkable achievement (with 100% success ) was made in tracking out the complete procedure for controlling the oxidative browning in the explants obtained from mature trees of four selected cultivars of apple (Rizvi et al., 2007, 2009). This involved selection of young twigs from well irrigated mature trees, their collection in early morning, double sterilization (pre-chilling and post-chilling), overnight chilling in kinetin solution (15µM), reduction in salt strength to half, addition of PG (10pM) in the medium and regular transfer of the explants to fresh medium of same composition for first few days. There is no such report available which describes this procedure for preparation of apple explants. However, Conger (1987) and Hu and Wang (1983) have suggested regular transfer of explants onto fresh nutrient medium of same composition for few days. Machado et al, (1991) have found addition of 8hydroxyquinol sulphate (8-HQS 200ml of 0.1% solution more effective. Bhat and Chanda] (1991) have suggested sealing of cut ends of explants with wax prior to inoculation. Dalal et al. (1992, 2006) have employed forced shoot tip culture method. Kumar and Kumar (1996) suggest treatment of explants with anti-ox1dants like Citric acid 150mgL prior to inoculation and rinsing sterilized explants with Diethyl-dithiocarbonate (DIECA $2gl^{-1}$) prior to inoculation. The highly effective

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role of Phloroglucinol (1OμM) in controlling oxidative browning by explants from mature trees in present study is in action with the outcome the work by Jones (1976) and Wilson and James (2003) but contradicts the observations of Snir and Erez (1980). Jones (1976) observed increase in shoot number, length and weight when PG was added to MS medium but elimination of IAA stopped proliferation of shoots. IAA was found ineffective in shoot proliferation in present studies. Wilson and James (2003) supported use of PG for shoot tip culture in Queen Cox cultivar. Contrary to these and our findings, Snir and Erez (1980) reported no impact of PG for shoot proliferation. Appropriate strength of nutrient medium is another important requirement for successful culture of apple explants. As most workers have used MS (1962) medium with full salt strength the present work was also started with same medium strength, but it did not yield good results probably due to hypertonic effect. Reduction in the salt strength to half was a major success in present studies as MS (1962) medium with half strength of its major and minor salts perhaps provided all essential requirements in balanced quantity. This finding corroborates with the work of Werner and Roe (1980) and Bartish and Korkhovoi (1997). Infact Bartish and Korkhovoi (1997) also suggested reduction in the strength of salts to onethird for promoting caulogenesis from leaf discs. In present observation, it was noticed that reduction in the strength of salts to half was ideal for promoting direct shoot multiplication in most explants and one-third strength resulted in the development of light green hydrated shoots. These findings confirm the earlier findings of Fei and Xue (1981), Le (1985), Wang et a1. (1985), Standardi (1985), Zimmerman (1985a and b), Wang (1990), Xue and Niu (1990), Stimart and Herbage (1993), Hoffmann et al.(2001) and Zhang et a1. (2010). However, it contradicts the findings of Cheema and Sharma (1983) who observed that half strength MS medium favoured the development of highly hydrated shoots, which were sensitive to injury and initiated much basal callusing. Choice of explant represents another essential requirement for tissue culture of apple. In present work, a number of types of explants were used to exploit their morphogenetic potential most of which showed satisfactory results. Best results in terms of applied aspects were obtained from shoot tips of mature trees as they can yield true to type plants through direct multiplication. This investigation stands continued by a number of recent works (Correa et al., 1990; Lankes, 1990; Lee et al., 1990; Sriskandarajah et al., 1990; Wang, 1990; Arello et al., 1991; Bhraldi et al., 1991; Machado et al., 1991; Orlikowska, 1991; Webster et al., 1991; Caboni et al., 1992; Moncousin et al., 1992; Puente and Marin, 1992; Uosukaimen, 1992; Harbage et al., 1993; Schuch and Peters, 1993; Stimart and Herbage, 1993; Sutter and Luza, 1993; Modgil et al., 1994; Yapes and Aldwinekle, 1994a & b; Niu et al., 1995; F erradini et al., 1996; He Shu Tao et al., 1996; Usek, 1996; Drought, 1997; Klerk et al., 1997; Marga et al., 1997; Bhat et al., 1999; Zhul et al., 1999; Hoffmann et al., 2001; Chakrabarty et al., 2003; Hao and Deng, 2003; Wilson and James, 2003; Dalal et al., 2006; Bahmani, et al., 2010) who have preferred use of shoot apices as explants for clonal micropagation of apple. One of the most important factors governing successful culture of apple explants is the selection phytohormones and their use in appropriate concentration and combination. In present investigation, best results were obtained when MS (1/2) medium was augmented with BA for shoot proliferation, IBA for effective rooting and GA3 for callus proliferation. Proliferation of shoot tips and axillary buds from mature trees of apple was found to be cultivar and season dependent. Maharaji and Chambura cultivars were found to have high regeneration potential than Ambri and Golden Delicious cultivars. Explants selected in spring and in early morning were found more vigorous than those selected in late summer, autumn or in aftemoon. These findings in present research are in conformity with the reports of Shih-feng et al. (1983) and Ivanova (1989). In present studies, adult phase explants were found to proliferate mostly through axillary shoots in initial cultures while seedling explants produced axillary as well as adventitious shoots at basal ends. Abbott and Whitely (1 976) have also published similar results. Literature in hand on apple reveals that explants from in vitro raised seedlings have higher organogenetic potential when compared with those obtained from mature trees. Present findings run parallel to it whereby seedling based explants have shown three times greater organogenetic potential than adult tree based explants. This is; however, of little practical importance as plants raised from seedlings in field never yield fruits unless they are

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subjected to grafting. In present work direct shoot multiplication in the shoot tips from mature trees of the four selected cultivars was achieved when the medium was fortified with BA (4.0-5 .0µM) alone, BA(4.0-5.0 pM) + PG (10pM) and TDZ (4.05.0µM) + PG (10µM) but best results were observed only under the influence of BA(4.0-5.0 11M) + PG (10µM). Thus results achieved on the culture of shoot apices run parallel to the findings of James and Thurbon (1979, 81), Zimmerman and Broome (1981), Zimmerman and Fordham (1989) and Modgil er a]. (1994) but contradicts with the findings of other researchers. Some researchers like Saito and Suzuki (1999), Sharma et al. (2004) and Nabeela et al, (2009) have succeeded in inducing direct shoot multiplication in the shoot apices of some apple cultivars under the influence of TDZ and have reported BA to be less effective in comparison to TDZ. On the other hand, Welender and i Huntrieser (1981) and Caboni et a1. (1992) have reported direct shoot. In present work Kn was found to play passive role in all apple cultures expect for pre-inoeulation preparation of explants from adult trees but in contrary Abbott and Whitely (1976) have found KN (0.5-4.6µM) to be effective for multiple shoot formation which differs with the present studies. Similar observation was reported by Lundergan and Janick (1980). However, researchers like Messer and Lavee (1969), Chong and Taper (1972, 1974a and 1974b), Walkey (1972), Kubiki et al. (1975), Coffm et al. (1976), and Mehra and Saroj (1979) have instead reported callus formation under the influence of KN. Use of BA(1µM) in present work was found to be too low to induce callus formation or morphogenesis in any apple explant as against Pech (1975) who reported BA (0.4pM) to be effective for callus initiation from different apple tissues. In fact callus formation from basal ends of shoot apices on MS + NAA in present work confirms the results of Coffin et al. (1976). BA (4-5µM) was registered in present work to be most effective for multiple shoot formation and shoot proliferation. Higher BA concentration was found less effective. In contrast, Yapes and Aldwinekle (1994) have reported BA (22.2µM) effective for shoot proliferation whereas Huth (1978) found that BA (8.9pM) + NAA (l.lpM) was best for establishment of very small (0.2mm) Jonathan shoot apices. Lane (1978) reported reduced shoot proliferation in Macspur meristem tips when the medium was supplemented with NAA or $GA_3$ which supports present similar findings. It seems that the eXplants used in present investigation had either high level endogenous cytokinins in comparison to those used by Yapes and Aldwinekle (1994) or less auxin / cytokinin ratio in comparison to those used by Huth (1978) and thus less concentration of exogenous BA was needed to counter the impact of endogenous phytohormonal combination. Use of TDZ in present studies did not yield better results than BA which was found to be much more effective. In contrast to this, Van Nieuwbreek et al., (1986), Fasiola et al (1989), Theiler and Theiler (1990), Dobzanski et al. (1991, 92, 2002), Diagny et al. (1996), Saito and Suzuki (1999) and Sharma et al. (2004) have reported TDZ to be more effective than BA for shoot proliferation and induction of multiple shoot formation from the explants of mature trees. While studying the effectiveness of different cytokinins in stimulating shoot proliferation Lundergan and Janick (1980) reported BA to be most effective, Zip to be least effective and KN to be intermediate. Moreover, shoots produced on medium containing BA (13.2-22.2 µM) were stunted and normal growth could be obtained by transferring the cultures to medium containing Zip or BA (4.4µM) + IBA (4.9pM). Use of BA in present study was found to have high stimulating effect on induction of caulogenesis. The optimum concentration of BA for promoting shoot formation was found to be 4-5µM. TDZ was found to be less effective for the induction of multiple shoots. This contradicts with the findings of Shanna et al. (2004) who have reported TDZ as most effective in inducing direct shoot multiplication in Ambri cultivar of apple. Golden Delicious shoots produced from in vitro grown seedlings took four to five weeks to develop multiple shoots. The shoots when subcultured further developed multiple shoots only in three-four weeks. In third subculture shoots expressed caulogenetic potential only in two weeks. The previous investigations on Delicious apple cultivars have also shown similar results which authenticate present results. While working on axillary buds of apple Pieniazek and Jankiewiez (1966), Powel (1970), Dutcher and Powel (1972), Byeong et a1. (1987), Chen and Evans (1990), Jasik et al. (1997) have observed multiple shoot formation under the influence BA (1.0.4.0µM). In present work shoot proliferation and multiple

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shoot formation could be observed only under the influence of BA (4.0-5.0µM) and TDZ (both in presence as well as absence of PG (10µM). However best response was observed only when the medium was augmented with BA (4.0-5.0µM) + PG (lOµM). Low concentrations of same phytohorrnones (2.0-3.5µM) induced callus formation and no response was seen under very low concentrations of BA (0.5-1.5µM). It was also observed that axillary buds of all the four selected cultivars of apple responded best when the twigs from mature trees were cut, surface sterilized and then kept in vertical position with their cut ends in KN (20µM) solution to force dormant buds to sprout under laboratory conditions. Present work on stem segments from mature trees revealed callus formation from the basal ends of stem segments on MS medium supplemented with low BA (3.5-4.0µM) concentration but multiple shoots were formed at both the cut ends under the influence of higher BA (4.55.0µM) + PG (l0µM). Thus BA (2.2µM) reported by Chen et a1. (1979) was found to be very low and BA (22.0µM) reported by Welender (1988) was found very high for inducing callogenesis or caulogenesis. Chen et al.segments on MS medium augmented with BA (2.2µM) or NAA (10.7µM) and casein hydrolysate (116 mg/l) which confirms our findings. Similar results reported by Saad (1965), Fuji and Nito (I972), Chong and Taper (1972), Evaldson (1985), Gladyshava (1987) and others also corroborate with our findings on mature tree stern segments. Hicks (1987) reported root formation from stem cuttings under the influence of low concentrations of IBA. On the other hand, Welender (1988) reported shoot formation at the cut ends of stem segments of five apple cultivars when the medium was fortified with BA (22.0µM). Pawlicki and Welender (1992) reported that BA strongly inhibited in vitro rooting of stem discs of apple and IBA promoted it. The results of leaf segments /discs of the four selected cultivars depicted direct multiple adventitious shoot formation along the margins of the explants on MS (X1/2) medium augmented with BA(5.0µM)+PG (10µM) or $GA_3$ (5µM). These results coincide with the results of Fasiola et al. (1989), Predieri et al. (1989), Ding and Chao (1992), Dobranski, et al. (1992), Durham et al. (1994), Aping and Fang (1996), Ding (1996) and F erradini (1996), Ding and Wang (1996), Wilson and James (2003), Modgil et al. (2005) and Dandekar et al. (2006) who noticed direct adventitious shoot formation along the cut edges of leaf segments obtained from the mature trees of different apple cultivars under the influence of moderate BA concentration. Slightly lower BA (4.0µM) concentration without or with PG induced callus formation in present studies. Contrary to it Gerson (1992) reported direct caulogenesis and embryogenesis on MS medium fortified with BA (4µM) + NAA (1µM) on Gala apple leaf discs. But as far as callus formation on leaf segments is concerned results run parallel with the findings of Gladyshava (1987), Da ka Dong (1995) and Martins et al. (2001) who reported the same results under the influence of low BA concentrations. Mehra and Saroj (1979) and Liv et al. (1981,83a, Saroj, 1979; Kouider etal., 1984; Rubos and Pryke, 1984; Joung et al., 1987; Stanys, 1992; Wallin er al., 1995; Wang et al., 1996 and Shanna et al., 2004). Mehra and Saroj (1979) reported root and shoot formation through callogenesis while culturing seeds of Golden Delicious apple cultivar on MS and White's medium, supplemented with NAA (ppm), IAA (5ppm) and CH (200ppm). Recently Shanna et al. (2004) reported development of axillary shoots from the seeds of Ambri apple on MS medium supplemented with BA or TDZ. TDZ (2.2µM) was found more effective than BA. Present work, has further revealed that formation of single root and shoot from a cotyledon free embryo or embryo with cotyledons occurs only on basal medium. Under the influence of BA (4.0-5.0µM) +PG (10µM) multiple shoot formation takes place. However, while working with Delicious embryos Kouider et al (1984) noticed that culture of tigellum or complete embryo with cotyledons yields single root and single shoot in all the cases. Our work shows synergism with their work in some respects like rapid germination potential and breaking of dormancy in Delicious seeds after removing testa and tegmen. Besides, immediate access to phtohormones for the culture of immature embryos was also found essential. Current research work also revealed MS (1/2) + BA (4-5µM) + PG (l0µM) to be the best medium for callus differentiation. This medium, however, promotes indirect caulogenesis only. Rhizogenesis was promoted in subcultured callus derived shoots by IBA (2.5µM). Mehra and schdeva (1979) and Liu et al (1981) observed leaf, root and shoot regeneration from the callus produced by different explants of Golden Delicious apple on White's medium containing

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NAA (4ppm) +KN (2ppm) + $GA_3$ (Ippm) +Casein hydrolysate (15%). Thus our results are contradictory to earlier work by Mehra and schdeva (1979). However, anthocyanin pigment formation in the cells of callus reported by Mehra and Saroj (1979) was also observed in present work also.Previous work on isolated cotyledons of different apple cultivars has depicted that they also show a strong organogenetic potential when cultured in presence of BA and IBA but develop callus under the influence of other auxins and Kn (Mehra and Saroj, 1979; Wu et al., 1997; Wang et al.,1980; Liu et al., 1981, Kouider et al., 1984; Rubos and Jannet, 1984; Stanis and Gyalvonauskis, 1988; Sniska, 1988; Gerson, 1992; Stanys, 1992; Ding et al., 1995; Wallen et al., 1995; and Sharma et al., 2004). These findings corroborate with our findings as both callus and multiple adventitious shoots were raised from the isolated cotyledons of all the four selected cultivars of apple under the influence of different phytohormones. Callus formation was seen along the margins of the isolated cotyledons under the influence BA (5µM) and $GA_3$ (5µM) and multiple adventitious shoot development was noticed when the medium was fortified with BA (5µM) +PG (l0µM).

In vitro studies of some apple cultivars namely Ambri, Golden Delicious, Chambura and Maharaji of Kashmir valley have helped to draw several significant, reliable and practical conclusions that if applied on industrial scale after refinement could help boasting apple production in J&K state. They are:-

*A.* Selection of young twigs from well irrigated mature trees, their excision from parent plants in early morning was found significant.

*B.* Overnight chilling of explants in kinetin solution (lS-ZOµM) at 4ºC in a refrigerator after initial but before final sterilization was found to be highly effective for establishment of primary cultures.

*C.* Double sterilization .of explants first before chilling and second after chilling helped in gaining complete control over contaminants and infectious agents.

*D.* Removal of iced coat in the seeds of all the selected cultivars after their chilling at 4ºC in a refrigerator prior to inoculation was found to be obligatory for controlling hidden bacterial contaminants.

*E.* Seeds Without seed coat showed rapid response but those with intact seed coat showed slow response for germination.

*F.* Satisfactory sterilization was achieved by using $HgCl_2$ (0.1%) in different time durations for different explants.

*G.* Browning of .media after “inoculation of explants from mature _trees, which 15 the biggest hurdle in in vitro culture of woody plants causing death of explants, was overcome by pre-inoculation chilling of the explants in Kn solution (20µM), addition of PG (l0µM) in the medium and regular transfer of explants onto fresh nutrient media of same composition for first few days.

*H.* Severe contamination in the medium even after establishment and several subcultures due to hidden infectious agents was effectively controlled by cutting of basal ends of the affected shoots on the hood of laminar air flow cabinet, dipping their cut ends in ethanol (90%) for 10 seconds and then their inoculation onto fresh medium.

*I.* The concentration of henolic exudates releasedby the explants from mature trees was foun to show inverse relation with growth rate of an explant.

*J.* Reduction of salt strength in MS medium to half was found essential for successful in vitro culture of all apple explants.

*K.* No prominent growth was observed in explants on basal medium (in the absence of phytohormones) of all explants used from mature trees for in vitro studies in all the four selected apple cultivars, shoot apices were found to show best response.

*L.* Degree of callus formation and regeneration was found to be hormone dependant.

*M.* Among cytokinins used BA was found to be most effective for shoot proliferation and multiplication.

*N.* Among auxins lBA was found to have significant influence on the induction of rooting.

*O.* Gibberellins ($GA_3$) could induce callus formation in the explants. Organogenesis was not noticed in any explant under the

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influence of $GA_3$.

*P.* BA (4-5µM) in association with PG (10µM) was found ideal for culture establishment and induction of direct shoot multiplication.

*Q.* The explants obtained from the mature trees of Ambri and Golden Delimous cultivars established well on MS (X½) + BA (5.0µM) + PG (10µM) where as those of Chambura and Maharaji cultivars established well on the same medium augmented with BA (4.0µM) + PG (l0µM).

*R.* Axillary buds from mature trees responded well only when they were taken as sprouts from cuttings kept in KNsolution under laboratory conditions.

*S.* Response of the explants for organoenesis from mature trees of all the selected cultivars was noticed either tough direct adventitious shoot formation or indirectly through callus formation.

*T.* Use of TDZ did not yield any significant result.

*U.* IBA (2.5µM) in association with PG (10µM) was found to be optimum for Induction of adventitious rooting in the shoots raised in vitro.

*V.* In vitro response of the four selected apple cultivars to different phytohormonal concentrations did not show much difference.

*W.* The survival rate and percentage of response was found to vary to some extent in different app 6 cultivars. Among the four selected cultivars, Maharaji was found to show highest reproductive potential, followed by Chambura, Golden Delicious and Ambri cultivars.

*X.* Reduction in the strenfgth ofmajor and minor salts in MS medium to one fourth resulted in the formation of hydrated shoots.

*Y.* Addition of PG was found to be indispensible for shoot proliferation and rooting of shoots in all the cultivars of apple under reference.

*Z.* Triple combination of auxins IAA (lµM) + NAA (lµM) + IBA (lµM) was found to promote callus formation at basal end in subcultured shoots.

*AA.* Explants from seedlings showed far better response in comparison to the explants from adult trees.

*BB.* Callus from any explant could differentiate in presence of high cytokinin and low auxrn concentration.

*CC.* Callus from the explants of adult apple trees in all the four selected cultivars was yellow 15h white, loose and friable while that from in vitro developed explants was generally compact, nodular and greenish in colour.

*DD.* Callus produced by the explants from mature trees showed less organogenetic potentlal than that produced by the explants from in vitro grown seedlings.

*EE.* The potential for shoot proliferation and direct shoot multiplication was found to 1ncrease after every subculture 1n all the selected cultivars.

*FF.* Direct multiple shoot formation was observed from the explants of all the selected cultivars on MS (1/2) + BA (5µM) + PG (10µM).

*GG.* Few successful trials on in vitro grading among different apple cultivars (under investigation) for the study of histocompatibility have opened new doors for further research.

*HH.* The results observed in all the selected apple cultivars were hormone oriented.

*II.* Testa free seeds of all the cultivars followed normal negatively photoblastic and epigeal mode of germination on basal medium and in presence of low BA concentration but became fleshy and callose with thick green cotyledons green and multiple shoots at shoot pole under the influence of BA (25µM) + IBA (5µM).

*JJ.* The microplants obtained through in vitro means hardened well when transplanted into sterilized soil mix (Soil-Peat 1:1).

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*KK.* The survival rate of the microplants under laborato conditions obtained through mature tree explant culture (MTEC) was found to be 85% and 9500 in those obtained through seedling born explant culture (SBEC).

*LL.* Among all the explants used shoot tips showed best response for the production of true to type plants.

*MM.* Among the four selected cultivars Maharaji cultivar showed the best response where as Golden Delicious

*NN.* Shoot tips of Ambri and Golden Delicious cultivars established well and also responded best in terms of direct shoot multiplication on MS (1/2) + BA (5pM) + PG (10µM).

*OO.* Shoot tips of Chambura and Maharaji cultivars established well and also responded best in terms of direct shoot multiplication on MS (1/2) + BA (4µM) + PG (10µM).

*PP.* Increase in BA concentration from 4.0µM to 4.5µM and 5.0µM not only reduced percentage of response but also maximum average shoot number in Chambura and Maharaji cultivars.

*QQ.* The maximum average shoot number produced per subculture of each shoot from mature tree explants was found to be 34±0.82, 38±0.82, 42±0.71 and 48±0.88 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively the highest being in the Maharaji cultivar.

*RR.* The average survival rate of in vitro raised microplants was recorded to be 85% in Ambn, 90% in Golden Delicious and 95% m Chambura and Maharaji cultivars respectively.

Apple is rosaceous pome fruit grown in temperate areas of the world for its high economic value. It is chiefly relished by people as fresh fruit but processed products like apple juice, canned apple sauce, apple jam and apple butter are also important table delicacies. Dehydrated apples, apple flour, apple dumpling, charoset (apple relish), apple haystacks are other important commercial products. The fruit contains appreciable quantity of sorbitol, sugars, organic acids and vitamins. It also has high medicinal value and serves as a stimulant for heart and as purgative, prevents constipation, reduces incidence of dental caries, helps to control obesity and supplies extra energy for heavy exercise. The pulp of apple fruit provides phytochemicals which serve as strong antioxidants and reduce the risk of some cancers, cardiovascular diseases, asthma, and diabetes. They also prevent oxidative stress and delay ageing. The other health benefits include better development of mental faculties. Apple trees when raised from seeds never produce quality fruits unless they are subjected to grafting. The traditional methods of propagation are time consuming, laborious and involve a lot of cost besides being skilful. Present investigation on in vitro studies of Ambri, Golden Delicious, Chambura and Maharaji apple cultivars has been taken in hand with an aim to develop complete micropropagation protocol for applying it in apple industry so as to boost the economy in the state. Explants were taken from 40-50 year mature trees. Shoot apices, axillary buds, nodal stem segments and leaf discs were collected for each trial from mature trees during early morning. Young twigs were cut out and placed in polyethene bags containing moist cotton. After washing them thoroughly under running tap water for half an hour they were sterilized in different sterilants. Double sterilization method was found to be highly effective. It involved initial sterilization of the explants obtained from mature trees in 0.1% Mercuric chloride, soon after thorough washing and final sterilization in same sterilant (0.05%) after chilling. Chilling of the explants for at least 24 hours was found highly effective in boasting culture establishment. Refrigeration of seeds from fresh fruits at 4°C in $GA_3$ solution for 7-14 days after initial sterilization was found essential for induction of germination. Besides, removal of seed coat before inoculation was also observed to be essential to promote quick growth in pre-germinated seeds. It also reduced the chances contamination of the medium by infectious agents hidden in the seeds. The sterilized explants were found to respond better on Murashige and Skoog's (MS) (1962) medium than on White's (1943) medium, Quoirin's medium (1972) and Woody Plant Medium (WPM Lloyd and Mc.Crown, 1980). However, results on full strength salt formulations of MS medium were much inferior to the strength when reduced to half which decidedly yielded much better results. Besides, augmentation of the medium

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with different cytokinins (BA, KN and TDZ), auxins (2,4-D, IAA, NAA and IBA), gibberellin ($GA_3$) and pholroglucinol (PG) in different concentrations and combinations yielded encouraging results. Throughout experiment, incubation room was kept completely aseptic and most explants were cultured under cool white light with 1500-3000 Lux light intensity. Temperature of the room was maintained between 22-28°C with 60-7 0% relative humidity. Exudation of phenolic compounds by explants (through their cut ends) especially those from mature trees are the biggest problem with the in vitro culture of apple. The exudates, after leaching out into the medium, not only turn it brown but being deleterious also kills the explants. A great success was achieved in controlling same by developing and applying a modified technique which includes selection of well irrigated trees as source of explants or using forced bud sprouts obtained after chilling branch cuttings of mature trees Overnight and then placing them vertically in water containing KN (15-20µM) Until buds sprouted, chilling of explants overnight (24hrs) in KN (15-20µM) before inoculation, reduction in the strength of MS major and minor salts in nutrient medium to half, regular transfer of explants onto fresh nutrient medium of same composition at least three to five times during first few days and addition of PG (l0µM) in nutrient medium. Shoot apices (0.5-1.0cm long) from mature trees of all the four cultivars responded only when the medium was augmented with cytokinins like BA, KN and TDZ (a compound having cytokinin like activity). It was seen that a maximum of 30% shoot apices could establish themselves when the phytohormones were used in the absence PG. Addition of PG (10µM) to medium containing BA (0.5-5.0µM) not only increased the survival rate but also powered culture establishment. Lower concentrations (0.5-3.0µM) of BA favoured callus formation at out ends in all cultivars but higher concentrations (4.0-5.0µM) induced direct shoot multiplication. The maximum average shoot number produced per subculture of each shoot was found to be 34±0.82, 38±0.82, 42±0.71 and 48±0.82 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively the highest being in the Maharaji cultivar. Shoot tips of all the selected cultivars obtained from in vitro raised seedlings also responded in the similar way. They readily established best on MS (X½) +BA (4.0-5.0µM) + PG (10µM) with cent percent response and also showed direct induction of multiple adventitious shoots at their basal ends. The maximum average shoot number produced per subculture of each shoot was found to be 38 ± 0.81, 39 ± 0.80, 50 ± 1.35 and 52 ± 1.75 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively again highest recorded in the Maharaji cultivar and higher than the number obtained from the shoot apices of mature trees in all selected cultivars. Axillary bud sprouts from mature trees of all the four selected cultivars were inoculated on MS (X½) medium with different hormonal concentrations and combinations. Best response was again observed on MS (X½) medium supplemented with BA (4-5nM) and PG (10µM). The explants took five weeks for establishment and then started proliferation of axillary shoots. Subculture of the shoots helped in getting multiple adventitious shoots. The maximum average number of shoots per subculture of each shoot was found to be 28±0.45, in Ambri, 29±0.80 in Golden Delicious, 30±0.72 in Chambua and 31±0.80 in Maharaji cultivar. Axillary buds from in vitro raised seeds of all the selected cultivars were also cultured under the influence of different cytokinins with or without PG. The explants produced callus when the medium was fortified with BA (2.0-3.5µM), BA (2.5-3.5µM) + PG (10µM), TDZ (2.0-3.0µM) or TDZ (3.0-5.0µM) + PG (10µM). Direct multiple adventitious shoot formation was registered to be the best when the medium was supplemented with BA (4.0-5.0µM) + PG (l0µM) with a maximum average number of 32±0.88, 34±0.65, 34±0.78 and 36±0.66 shoots per subculture per shoot in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively. Nodal stem segments taken from juvenile branches of mature trees of all the selected apple cultivars also produced callus under the influence of BA (3.0-4.0µM), KN (5.0µM), KN (5.0µM) + PG (10µM) and BA (5.0µM) + KN (5.0µM) +PG (10µM) and developed multiple adventitious shoots when the medium contained BA (5.0µM) or BA (4.0-S.OµM) + PG (10pM). The maximum average number of shoots produced per subculture of each shoot was calculated to be 12±0.45, in Ambri, 12±1.75 in Golden Delicious, 14±1.85 in Chambua and 14±1.54 in Maharaji cultivar. Nodal stem segments from in vitro raised seedlings of the under reference cultivars were also grown on MS medium fortified with

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different hormones. The explants produced moderate, greenish and compact callus at cut ends when the medium was supplemented with BA (3-4µM), KN (5.0µM) or Kn (4.0-5.0µM)+PG (10µM). Adventitious shoot formation was observed at both the ends on MS (X½) + BA (4.0-5.0µM) + PG (10µM). The maximum average shoot number per subculture of each shoot was found to be 32±0.78, 32±0.82, 28±0.87 and 38±0.62 in Ambri, Golden Delicious, Chambura and Maharaji cultivars respectively comparatively higher in Chambura and Maharaji cultivars than Ambri and Golden Delicious cultivars. While examining the cultures of leaf segments from mature trees of all the selected cultivars it was seen that they responded either by producing callus along cut edges or by forming adventitious shoots along margins. Yellow loose, moderate callus developed along the margins of leaf segments mature trees of all the cultivars under study when they were cultured on the MS (X½) medium fortified with BA (5µM), $GA_3$ (3-5µM) or $GA_3$ (3-5µM)+ PG (10µM). Ambri and Golden Delicious leaf segments produced callus of same nature on MS (X½) + BA (4µM) + PG (10µM) but Chambura and Maharaji leaf segments developed adventitious shoots. Best response in all the four cultivars was noticed on MS (X½) + BA (5µM) + PG (10µM) in terms of adventitious shoot proliferation. The maximum average shoot number was found to be 10±0.88 in Ambri, l4±0.59 in Golden Delicious, 16±0.72 in Chambura and 16±0.74 in Maharaji cultivar. Seedling born leaf segments showed dedifferentiation of cells along their cut edges and produced callus when the medium was fortified with $GA_3$ (4-5µM), but produced multiple adventitious shoots under the influence of BA (4-5µM) + PG (10µM). Root Shoot Transitional Regions of all the four cultivars were also cultured on different media augmented with different hormones and were found to respond better on MS (X½) medium fortified with BA (3.5-5.05µM) + PG (10µM). Callogenesis was noticed at both cut ends MS(X½) + BA (5pM) + IBA (2.5µM) but adventitious shoots were noticed under the influence of BA (5.0µM) + PG (10µM). Root segments obtained from in vitro raised plantlets of all the selected cultivars were cultured on MS (X½) medium supplemented with different phytohormones in different concentrations to assess their morphogenetic potential. The explants responded well either by way of callus formation or through organogenesis. Callus formation on the surface was noticed when the medium was fortified with BA (4-5µM), KN (5µM), $GA_3$ (4-5µM) or BA (5µM) +KN (5µM) +PG (10µM). The callus produced was initially white and loose but later on turned green. However, augmentation of the same medium with BA (5µM) +PG (10µM) favoured direct adventitious shoot bud formation on the surface of the root segments. Root segments of Ambri and Golden Delicious cultivars showed 95% response with a maximum average number of 5±0.88 and 6±0.59 shoot buds but those of Chambura and Maharaji cultivars showed cent percent response the maximum average number of 9±0.72 and 6±74 shoots per subculture of each shoot respectively. Partially germinating, double sterilized and testa free seeds obtained from the freshly harvested fruits of all the selected cultivars were found to open cotyledons with no further growth on MS (X½) +IAA (4.0µM), IBA (4.0µM) or NAA (4.0µM) but followed normal growth pattern on MS (X½) +BA (4.0-5.0µM) +lBA (l0-2.5µM) +PG (10µM). Growth of root and shoot was arrested in the seedlings after opening their cotyledons under the influence of BA (4.0-5.0µM) + PG (10µM). Instead lateral swelling followed by multiple adventitious shoot formation was recorded on the same medium with 100% response. The shoots were subcultured individually for further proliferation and multiplication on same medium in the sixth week of culture period. Once sufficient shoots were produced some shoots were allowed to continue multiple shooting but most of them were isolated and subjected to rooting. Cotyledon free embryonal axes (tigella) excised from the seeds of all the selected cultivars were inoculated on a medium with or without growth supplements in different concentrations and combinations. Amongst auxins, explants responded to IBA (2-3µM) only by the way of low callus formation. Under the influence of cytokinins like BA (2.5-5µM) with PG (10µM) better results in terms of callus and multiple adventitious shoot formation were obtained. Cent percent response by way multiple adventitious shoot formation was noticed when the tigella were cultured in presence of BA (5µM) with PG (10µM). The shoots produced were subcultured several times until they attained sufficient number. The maximum average number of shoots produced was recorded to be 38±1.32 in Ambri,

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38±0.75 in Golden Delicious, 42±0.86 in Chambura and 44±1.25 in Maharaji cultivar.

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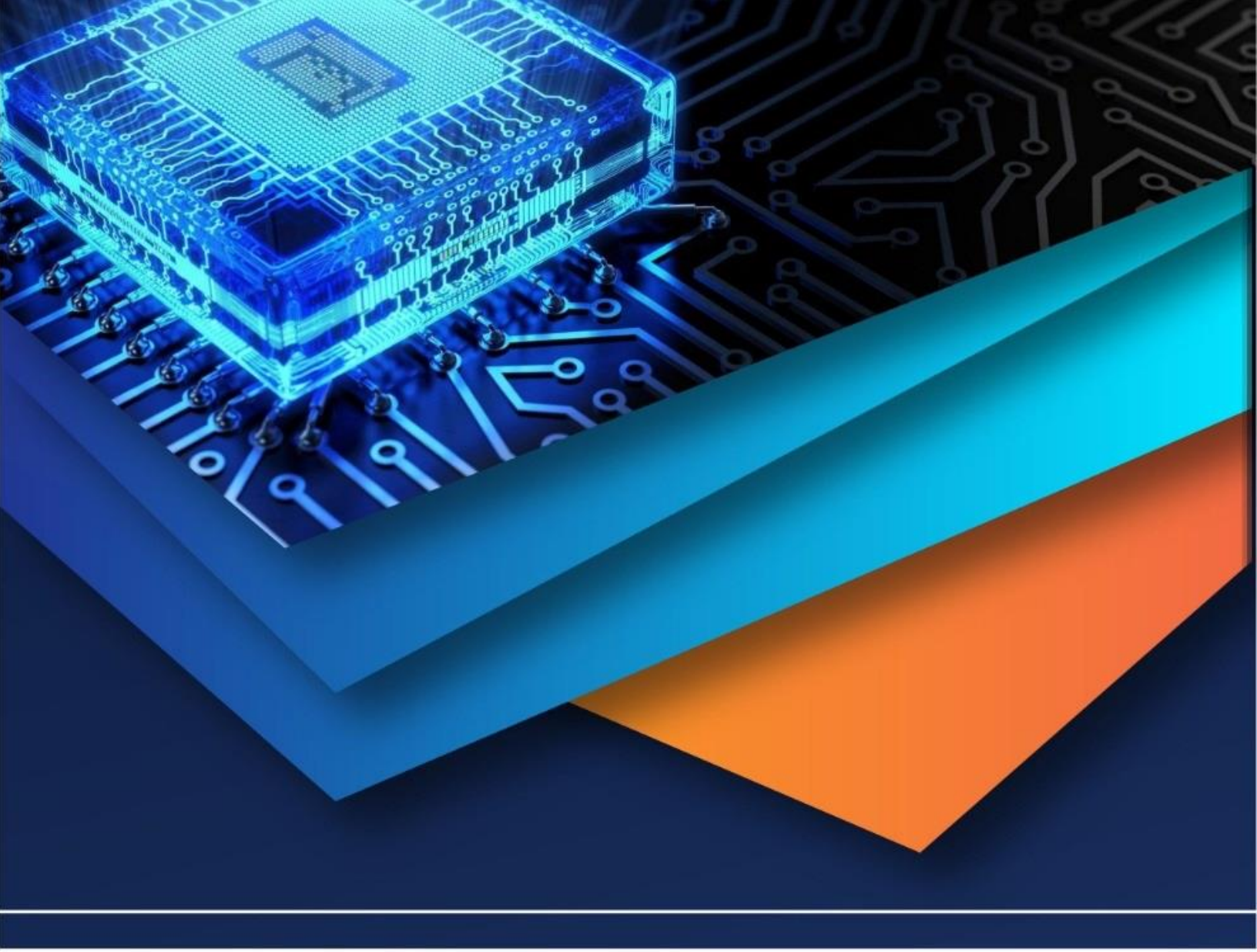

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