Discussion

The seeds of *Cucumis sativus* L. (*var*. Long Green) showed a near 90 to 92% germination. The emergence of radicle was treated as the germination. This percentage of germination under laboratory conditions overall can be taken as an assurance of the seed stock quality and its uniformity. Cucumber seed, seedling or its isolated cotyledons or hypocotyls have been successfully employed by other workers too also to ascertain physiological parameters in plant tissues (Shuab *et al*., 2013; Yu *et al*., 2009; Ding *et al*., 2007; Daayf *et al*., 1997; Sharma and Kaushik 1982). In the present study also the use of isolated cotyledons of cucumber (*Cucumis sativus*) with some modifications in preparation for bioassay was therefore, justified. The bioassay seems to provide some advantages as far as convenience and also performing and creating experiments under controlled conditions are concerned.

Cinnamates is a group of compounds which are synthetically available, but are predominantly being synthesized by the plants of almost all groups (Robbins, 2003). Cinnamates and/or its derivatives have therefore, been alleged of their ubiquitous presence in plants. These have been extracted from every plant part such as roots, stem, leaves, flowers, fruits and seeds (Choi *et al*., 2011; Hegab *et al*., 2008). Though, salicylates have now almost been established for their having growth and

developmental functions in plants and being recognized as plant growth regulators, the cinnamates despite their ubiquitous presence are yet to be identified as ones of these. This objective of the present work has very recently been put on the pedestal by Ghareib *et al*., (2010) and Firn and Jones, (2009) also. This therefore, seemed aptly justifiably motivating in undertaking the work.

Here a comparative study had been undertaken to ascertain whether, the cinnamate its natural *trans*- isomer alone or in conjunction with other PGRs can have some physiological and biochemical effect on the growth and developmental parameters using a photosynthetic tissue. Therefore, for comparison its effects had to be evaluated along with known established plant growth regulators both promoters and inhibitors included kinetin; auxin; gibberellic acid as promoter PGRs and abscisic acid, as a growth inhibitor. Various functions have already been ascribed to phenolics by many workers which have been referred to in the literature review section earlier. Amongst these reports by Buer *et al.,* (2010), Mukherjee and Kumar, (2007) and He and Lin, (2001) demand a specific mention here. These groups tried to emphasize the promotion role of phenolics especially with regard to their stimulation of IAA, GA_3 and kinetin activity. These reports overall can be taken as too few and far scanty. Therefore, the present work with cinnamates was taken up with a conviction that this work may add to the already existing scanty information regarding the role of phenolics in the physiological growth and development. Further emphasis for employing the isolated cucumber cotyledons as a bioassay is laid here. This is because the tissue is uniformly photosynthetic without any interference from root uptakes and/or endogenous production of PGRs to an extent of physiological effect. The effects therefore, produced could be predominantly those of exogenous PGR and/or cinnamic acid.

In the present study it was observed that at higher concentrations the cinnamate became inhibitory to growth and development of cotyledons and over time caused degradation to an extent of fatality. The standardization therefore, implied to limit the concentrations of cinnamate to the levels which could prove sustainable over more than 120 hours of floating on the treatments. Such inhibitory and toxic role of phenylpropanoids is well documented (Lima *et al.*, 2013; Jitareanu *et al.,* 2013; Chen, *et al.,* 2011; Huang and Bie, 2010).

The cinnamate was used alone and in combinations at various concentrations to see their interactive effects, if any, on the mobilization of certain tissue metabolites; activity of several enzymes; protein profiling; phytochemical constituent differences and phylogenetic/genetic variations.

The cinnamate independent or in combination with kinetin showed dose dependent fresh weight increase. This was independent of kinetin presence with lower cinnamate concentrations. The two later seemed to synergise the antagonistic effects when each being at highest concentration. Kinetin, affect fresh weight increase in cotyledons through cell expansion water uptake than by any substantial dry matter addition. Results seem to show that when in combination cinnamate probably takes over from kinetin in accumulating fresh weight due to increase in dry matter content of cotyledonary tissue which showed constant and continuous significant increases with the increasing cinnamate concentration by decreasing the moisture percent. This therefore, can lead to the more significant dry matter accumulation due to cinnamate than due to kinetin at every stage of treatment. The kinetin are known to increase fresh weight due to cell expansion resulted by water uptake than any addition of dry matter (Laloraya *et al*., 1986). Cinnamate is reported to act independently than interactively with kinetin. It is further reported that kinetin has no additive inhibition

or synergistic effect with cinnamate as far as dry matter accumulation or even metabolite mobilization in isolated cotyledons of cucumber is concerned (Shuab *et al.,* 2013). Therefore, the increase in dry matter due to independent action of cinnamate seems explainable. The toxicity in cinnamate and kinetin treatments at higher concentration produced toxicity whereas, the individual treatments not toxic. Therefore, the combined effects do seem synergistic in degradation process; probably by enhancing metabolic inhibition. A detailed further study to this effect can be suggested. Synoptically it is obvious that cinnamate improves dry matter accumulation *vis a vis* fresh weight, whereas, addition of kinetin depresses, IAA again reduces once increased, GA_3 again depresses and ABA reduces the fresh and dry weight contents. The increase in dry matter therefore, can be said the independent action of cinnamate, an observation first time reported in this work. Naturally occurring cinnamate compound have been reported to test just as auxins do (Yang *et al.,* 1999), though the mode of action by *cis*-cinnamate was different from that of auxin.

The cinnamate alone or in combination with gibberellic acid showed slight increase in both fresh and dry weight of cotyledons. Gibberellic acid superimposition even seems to antagonise and overcome the toxic effect due to highest cinnamate. It was not seen in kinetin interaction. Therefore, GA_3 was increasing the longitivity of the cotyledons even at highest concentration of cinnamate. The reason seems that gibberellic acid as already been reported to increase cell elongations and translocations across the membrane (Arteca, 1996; Stoddart, 1986;). Gibberellic acid here seems to increase the cinnamate transport enhancing the phenylpropanoid biosynthesis thereby, increasing the longitivity of the cotyledons even at highest concentration of cinnamate.

All the three photosynthetic pigments *viz.* chl. a, chl. b and total chl. showed increase at lower cinnamate doses upto suboptimal cinnamate concentrations. However, the highest cinnamate dose shows a sharp decrease in all the three pigment contents. Kinetin superimposition synergise the cinnamate effect on cotyledonary chl a. and total chl. content however, sharpens the loss of chl. b content. Auxin superimposition along with cinnamate doses shows small increase in all the three photosynthetic pigment contents. Gibberellic acid superimposition further enhanced the cinnamate responsive increase in photosynthetic pigments. Gibberellic acid even here antagonise the highest cinnamate dose concentration. Abscisic acid superimposition sharpens decrease in photosynthetic pigment contents due to cinnamate highest dose concentration, which otherwise shows increase. These observations are in consonance with those of (Mukherjee and Kumar, 2007; Wong *et al.*, 2005; Nutbean and Briggs, 1982).

Plant phenolic compounds have widely and more recently been reported to be substances stimulatory to plant growth and function as promoters (Ghareib *et al*., 2010; Hegab *et al*., 2008). Also other workers have reported the phenol responsive increase in mobilization of metabolites like carbohydrates, proteins and total phenolics (Shuab *et al.*, 2013; Talaat and Balbaa, 2010; Talaat, 2005; He and Lin, 2001; Zeng *et al.*, 2001). Further kinetin and salicylic acid (SA), a phenolic substance has been said to regulate plant growth and development by enhancing GA metabolism of plants (Mukharjee and Kumar, 2007). As the observation here conform to those cited above, it will be appropriate to say that effects on overall metabolite spectrum especially the sugars, proteins and total phenols are much more visible with clarity in one system. For example the cinnamate induced depressions at each concentration are overcome by IAA, GA_3 in each reducing, non-reducing and total sugar content levels

and ABA inhibition is overcome by cinnamate presence. Looking somewhat contradictory it needs an additional study to establish the cause of this observation due cinnamate. The phenol responsive increase in mobilization of carbohydrates, proteins and total phenolics are already reported (Shuab *et al*., 2013; Talaat and Balbaa, 2010; Talaat, 2005; Zeng *et al.,* 2001).

In the present study, sugar pool of the cotyledons, showed that, there is a constant, concomitant increase in the tissue reducing, total and therefore non-reducing sugar levels with the increasing doses of cinnamate irrespective of whether these are superimposed with any of the other plant growth regulators. Here again it therefore, seems that any dry matter accumulation is resulted by cinnamate than other growth regulators. In case of cinnamate and kinetin interactions there is a significant increase at lower concentration combinations. The two together decreases the sugar pool at higher dose combinations of both (Shuab *et al*., 2013). Auxin and cinnamate together showed uneven interference in sugar content. It seems that auxin interferences in cinnamate metabolism. Earlier cinnamate was thought to be an auxin analog therefore, showed fluctuations of sugar pool, when used in several combinations. Gibberellic acid and cinnamate together enhanced the increase in sugar pool significantly thereby, increasing the longevity of cotyledons by overcoming the effect of highest cinnamate dose concentration. Cinnamate and abscisic acid together showed that abscisic acid inhibitory responses are suppressed by cinnamate on the cotyledons at low concentrations.

The protein in the cotyledons in response to cinnamate showed an insignificant time bound increments which percentage wise too are insignificant. This effect of cinnamate is already reported earlier (Shuab *et al.*, 2013; Singh *et al.*, 1997). It seems that since the cotyledons are already provided with cinnamate exogenously

the endogenous synthesis of phenols and their concomitant enzymes and proteins are inhibited or are at low levels. Therefore, the meagre time bound protein increase seems obvious. Also phenolic substances sensed exogenously are known to be inhibitory for protein synthesis and therefore, show their presence at low levels throughout, whatever the treatments. More so, cinnamate is an established allelopathic compound (Chen *et al*., 2011; Chobot *et al*., 2009; Fuzita and Kubo, 2003). Kinetin superimposition sharpens the protein depressions at highest cinnamate concentration. Kinetin seems to be responding in eliciting the metabolic channeling here, something which is already known (Winkel, 2004). In cinnamate and auxin treatment combinations the auxin antagonise the time bound cinnamate responsive protein increments. Gibberellic acid superimposition showed insignificant increments over lower cinnamate concentrations. However, gibberellic acid could antagonise the depression in protein content caused due to highest cinnamate concentration. But in cinnamate abscisic acid combined treatments, abscisic acid synergise the protein increase due to cinnamate. Also, it seems here that cinnamate overcomes the growth retarding effect of abscisic acid, thereby increasing the protein content irrespective to abscisic acid superimposition.

The tissue phenol levels with cinnamate alone and in combination with all the four plant growth regulators keep marginally increasing with time in the cucumber cotyledons. This may be as a result of cinnamate uptake from the medium. It seems that other growth regulators may not be interfering with the cinnamate uptake by the tissue. It is reported that cinnamic acid has the ability to uncouple the energy transducing membrane and stimulate non-specific membrane permeability which allows the influx of the substances say kinetin and cinnamate across the cell wall and

membrane (Chambel *et al.,* 1999). This may explain the concomitant increases in total phenol level within the cotyledons.

Plant growth and development is determined by the mutual interaction between the plant growth regulators. The cinnamate in the present study showed synergistic and antagonistic interference with other plant growth regulators *viz.*, kinetin, auxin, gibberellic acid and abscisic acid, when used in several dose combinations. The fresh and dry matter contents; photosynthetic pigments; metabolite mobilization; carbohydrate fractions; protein expression; phytochemical constituents show considerable variability in isolated cucumber cotyledons. The results of this study to some extent provides a better understanding of cinnamate interactions with established growth regulators *vis a vis* biochemical and physiological processes in the bioassay employed in the study.

The application of cinnamic acid considerably increases the production of catalase and peroxidase. This may be an aspect of some kind of stress induction in the cotyledons. This type of observation due to cinnamate is reported by Yang *et al.*, (2006) on the tomato seedling root tips, where they explained that the plant material is forced to over synthesize O_2 and H_2O_2, hence is followed by a response of antioxidant enzyme systems. The stress explanation is corroborated by the presence of amino acid L-Proline in GC-MS phytochemical analysates where cinnamate alone is provided. L-Proline is established to accumulate in response to both biotic and abiotic stress by plants (Szabados and Savoure, 2010; Man *et al.,* 2011).

The ineffectiveness of cinnamate in the induction and infliction of higher PAL activity can probably be ascribed to inactivation of the enzyme due to the availability of cinnamate in the medium hence probably the feedback inhibition. The tissue being

provided with *trans*-cinnamate exogenously reduces the need to synthesise it endogenously, hence low PAL activity. The effects of *trans*-cinnamic acid on the expression of phenylalanine ammonia lyase gene family has been already worked out by Mavandad *et al.*,(1990) who showed that cinnamic acid or a derivative could act as a component in a regulatory feedback system operating at the level of phenylpropanoid gene transcription.

The HPLC profiling of the carbohydrate fractions are clearly indicative of that amongst these metabolites the sucrose which is the ultimate translocated carbohydrate has some role in *trans*-cinnamate induced changes in carbohydrates *vis a vis* other plant growth regulators. Infact Kim *et al.,* (2009) have suggested that the effect of sugar on plant metabolism, which is known to be similar to hormone – like signaling. It was metabolomically studied using *Melissa officinalis*. The metabolite profiles of this plant analysed by GC-MS reported 64 metabolites from various chemical classes including alcohols, amines, amino acids, fatty acids, organic acids and sugars. They have reported that metabolite profiling with regard to presence of proline and succinic acid, which are associated with the proline linked pentose phosphate pathway, the shikimic acid pathway and the biosynthesis of phenylpropanoids leads to an abundance of changes in primary and secondary metabolites. The difference in the work presented here being that in case of Kim *et al.,* (2009) the sucrose is exogenously applied and in work here the sucrose is increasingly produced in response to a phenylpropanoid *trans*-cinnamate which is then inducing various amino acids like proline, array of fatty acids, organic acids, alcohols and amines etc. as are evident from the GC-MS analysis in cinnamate treated cotyledons. The addition of various other growth regulators only limit to elicitation and/or addition of few more

compounds of these chemical groups of organic origin. Whether this is the case at *in vivo* level shall however, need a thorough further investigation.

SDS-PAGE analysis performed using MINI – PROTEIN (Biorad) apparatus on staining with Coomassie Brilliant Blue R-250 solutions followed by destaining shows that protein profiles do change with *trans*-cinnamate treatment. The profiles are dose dependent. There are differences in the polypeptide banding patterns between lower and higher cinnamate concentration treatments. The addition of other growth substances with or without cinnamates show effective and various banding profiles on the gels. This probably signifies that in case of green cucumber cotyledons under constant illumination do induce and/or reverse expression of proteins, hence showing differences within plant growth regulator treatments with or without cinnamate presence. This is indicative of an induction of active metabolism within the tissue. Hence the sequence as per this study therefore, infers that once cinnamate is provided, there is a range of metabolic changes induced through both ana and catabolic reactions. The differential induction is cinnamate dose dependent and also on the presence of exogenously applied growth regulator with or without cinnamate. This inference seems to be sustainable due to similar observations by Chen *et al.*, (2005). This therefore, should imply that cinnamate presence should either activate and/or deactivate cum suppress gene expression, inducing synthesis cum absence of both functional and structural proteins. This analysis however, does need an exclusive future study.

For the time as at present and through this study there is hardly any other conclusive study that could stand to testify cinnamate/s as a group of plant growth regulators on their own. However, the observations discussed here do at least drive one to a presumption that cinnamic acid *per se* has its own identity in effecting

growth and developmental characteristics of isolated cucumber cotyledons and these seem to be regulated both at the genomic, proteomic and metabolomic levels. Further characterization of this ubiquitous plant phenol and its derivatives as growth regulator/s will be established only once the system is operated on the whole plant basis than an isolated tissue system. In any case a beginning seems to have been made.

The RAPD analysis using nearly ten primers and studying their variational pattern on the gels showed a lot of heterogeneity in the DNA band pattern hence expression in response to cinnamates and combined cinnamate – plant growth regulators. The clustering has shown three treatment clusters and here too the cinnamate only cluster stands apart from other two clusters. This seems to be a significant observation in assuming an exclusive gene expression resulted by the cinnamate itself. Hence the statement in the above para that beginning seems to have been made is further substantiated.

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